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Investigation of Sugar/Polyols as Weakly Interacting Cosolvents and their Influence on Hardening of High-Protein Nutrition Bars

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INVESTIGATION OF SUGAR/POLYOLS AS WEAKLY INTERACTING COSOLVENTS AND THEIR INFLUENCE ON HARDENING OF HIGH-PROTEIN NUTRITION BARS

by

Sami Kadhim Hassan

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ABSTRACT

Investigation of Sugar/Polyols as Weakly Interacting Cosolvents

and their Influence on Hardening of High-Protein Nutrition Bars

by

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High-protein nutrition (HPN) bars (≥ 30% protein) have limited shelf life and become excessively hard during storage. Various mechanisms have been proposed to explain the hardening. The objectives of this research were to investigate the chemistry of HPN bar hardening and propose solutions for slowing it and improving bar texture.

In phase 1, HPN bars were made containing 34% whey protein isolate (WPI) or milk protein concentrate (MPC) powder, along with either sorbitol syrup or glycerol, and vegetable shortening or cocoa butter. Substituting MPC for WPI made the bars brittle and crumbly. Using glycerol initially made bars softer but accelerated hardening. Cocoa butter increased bar hardness because of its higher solid to liquid content. Most water (~99%) in HPN bars made using sorbitol syrup is present as bound water, with ~0.9% as intermediate water and ~0.1% as bulk water. During storage bound water increased ~0.02 g/100 g of solids while intermediate water decreased, suggesting changes in state of water taking place at protein surfaces. During storage, there were changes in protein conformation indicated by an increase (~4°C) in heat denaturation temperature of β-lactoglobulin and α-lactalbumin and a 15 to 40% decrease in denaturation enthalpy.

In phase 2, various bar formulations were tested involving different proportions of proteins, lactose, glycerol, and sorbitol syrup, as well as type of lipid component, and disulfide bonds inhibition. Decreases in bar hardening occurred when MPC and WPI and sorbitol syrup and glycerol were used in combination.

In phase 3, HPN bars made with 38% protein powder as a 50:50 combinations of WPI and MPC and with 20% of sorbitol syrup substituted with glycerol, had good texture and minimal hardening during storage. Bar hardening was not caused by phase separation of protein and sorbitol, Maillard browning, or formation of inter-molecular disulfide bonds. Minimizing bar hardening requires prevention of entropy-induced protein aggregation by masking hydrophobic regions on protein surfaces and preventing formation of extended protein networks. It is proposed that preferential exclusion of cosolvents causes glycerol to be oriented at protein surfaces such that its carbon backbone masks hydrophobic regions thus avoiding a decrease in entropy of water molecules.

(229 pages)

PUBLIC ABSTRACT

Investigation of Sugar/Polyols as Weakly Interacting Cosolvents
and their Influence on Hardening of High-Protein Nutrition Bars

Sami Kadhim Hassan

The Western Dairy Center (WDC) at Utah State University demonstrated opportunities to improve the quality of high-protein snack foods that becoming more prevalent in western diets. Previously, such high-protein nutrition bars became too hard during storage and they had a limited shelf life, resulting in disappointment by consumers or loss of product as older products needed to be discarded.

With sponsorship as a doctoral student by the Iraq Ministry of Higher Education and Scientific Research and in conjunction with WDC researchers, an investigation was conducted into the chemistry of high-protein nutrition bars and how the various components (protein, carbohydrate and lipid) could be varied to improve their texture and prevent hardening during storage. This project developed a scientific understanding of why hardening occurs during storage based on the chemistry that control interactions on the surface of proteins in high-protein food systems. We further identified combinations of protein powders and carbohydrates that could be used to allow high-protein nutrition bars to be successfully stored for longer times and making it possible to increase the level of protein that can be in the bars.

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DEDICATION

I dedicate my dissertation to my passed away parents and siblings, Prof. Donald J. McMahon because without his help and encouragement this could not been successfully completed. Finally I want to dedicate this to my entire family, especially my wife Aswan and my sons Abdullah, Abdulrahman, Abdulmalwa, and Abdulali.

Sami Kadhim Hassan

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LIST OF ABBREVIATIONS

α-La = α-lactalbumin

β-Lg = β-lactoglobulin

BSA = Bovine serum albumin

CLSM = Confocal laser scanning microscopy

DSC = Differential scanning calorimetry

DTNB = Ellman's reagent 5,5-dithiobis(2-nitrobenzoic acid)

DTT = Dithiothreitol

EDTA =Ethylenediaminetetraacetic acid

ΔE = Color change of bar samples during storage

FITC = Fluorescein isothiocyanate

HPN = High-protein nutrition

HWPI = Hydrolyzed whey protein isolate

ΔH = Enthalpic change

MPC = Milk protein concentrate

MPI = Milk protein isolate

NTB^{2-} = 2-nitro-5-thiobenzoate anion

$NTSB^{2-}$= Disodium 2-nitro-5-thiosulfobenzoate anion

%RC = Relative changes

SDS-PAGE = Sodium dodecyl sulfate-Poly acrylamide gel electrophoresis

T_m = Denaturation temperature

WI = Whiteness index

WPC = Whey protein concentrate

WPI = Whey protein isolate

CHAPTER 1.

LITERATURE REVIEW

High-protein nutrition (HPN) bars are intermediate moisture foods that are part of the sports nutrition, muscle building, health supplement, and weight reduction markets of the food industry (Liu and others 2009). They provide healthy alternatives to conventional snacks because of the inclusion of 15% to 35% (wt/wt) of protein. In addition to protein they contain lipids and various carbohydrates including humectants/plasticizers such as glycerol and sugar alcohols such as sorbitol. Chocolate, sugar, flavorings, nuts, wafers, vitamins, minerals, dried fruit and fibers are added to enhance flavor, texture and nutritional value. The base components (protein, carbohydrate and fat) of HPN bars can be mixed into a dough that is soft, malleable, and easily formed into bars (Gallo-Torres 2003; McMahon and others 2009). Inclusion of high levels of protein in HPN bars results in adverse quality effects, in particular bar hardening, which can be become detectable after 2 to 3 mo and unacceptable after 4-6 mo (Taillie 2006). This hardening occurs without any moisture loss (Zhou and others 2008a, 2008b; Hogan and others 2012) as the bars are sealed to prevent drying. Such hardening causes consumer avoidance (McMahon and others 2009).

A wide variety of proteins are used in commercial HPN bars including whey proteins, soy proteins, caseinates, egg proteins, and gelatins (Liu and others 2009). Such use of multiple protein powders in commercial HPN bars suggests that mixtures of proteins are beneficial with respect to controlling hardness, compared with single protein systems. These can take the form of protein isolates (>90% protein), proteins

concentrates (<90% protein) and protein hydrolysates. The use of hydrolyzed whey protein has been found to maintain softer bar texture over time (McMahon and others 2009) but can be accompanied by a bitter flavor (Hogan and others 2012) and also promotes Maillard browning reactions (McMahon and others 2009).

Unfortunately, the production of HPN bars is rather empirical and innovations are limited by lack of scientific insight into the mechanism of hardening although various mechanisms have been proposed. These include aggregation of proteins following formation of intermolecular disulphide bonds and non-covalent interactions (Zhou and others 2008a, 2008b), Maillard reactions that result in protein polymerization (Tran 2009), moisture migration (Labuza and Hyman 1998; Liu and others 1991; Loveday and others 2009, 2010) and phase separation (McMahon and others 2009, Loveday and others 2010). A challenge is that formation and control of micro- and macro-structures in concentrated solid food systems is still poorly understood (Ubbink and others 2008; Purwanti and others 2010). In general, soft materials such as HPN bars, are thermodynamically unstable (Van der Sman and Van der Goot 2009) leading to age related, structural and textural change, such as hardening. Since manufacturers of HPN bars have little knowledge about either the mechanism behind bar hardening or the changes that occur during storage it is necessary to gain a better grasp of what is taking place as the HPN bars harden.

High-Protein Nutrition Bars Market

High-protein nutrition bars were once marketed to athletes and competitors, but today are formulated, marketed, and sold to the everyday consumer (Hutchinson 2009). They may also be marketed for meal replacement, athletic supplements, body building

aids, and balanced nutrition. Meal replacement HPN bars offer more balanced nutrition than snack or candy bars and provide satiety between meals (Book 2008). The market for nutrition bars in the United States grew to $3 billion in 2007 although the initial rapid growth in sales of HPN bars leveled out as interest in low carbohydrate diets dwindled, because of their higher cost compared to other food bars (Wright 2008). There were some trends related to fortification of food bars with protein or fiber and inclusion of organic or all-natural ingredients.

High-Protein Nutrition Bar Composition

High-protein nutrition bars are composed primarily of powdered proteins from dairy or plant protein sources and sugar- or polyol-based syrups. A lipid is added to provide malleability of the bars. Protein is an important component in HPN bars, and although there is no official standard, they typically contain 20 to 35% protein by weight. Chocolate, sugar, and flavorings create an appealing taste and flavor. Nuts, wafers, nuggets, etc. may be added for novel texture. Vitamins, minerals, and/or fibers are often added for enhanced nutritional value (Loveday and others 2009). Dairy proteins used in HPN bars (as shown on ingredient lists of commercial bars) include whey protein concentrate (WPC), whey protein isolate (WPI), hydrolyzed WPI, milk protein concentrate (MPC), milk protein isolate (MPI), calcium caseinate, sodium caseinate, casein and nonfat dry milk. Other proteins include egg white, soy protein isolate, and organic soy flour.

Additional ingredients include soy lecithin, dicalcium phosphate, sugar, and flavorings, vitamins, minerals, fiber and nuts to enhance texture, flavor or nutritional value. Besides the high-protein matrix other components include flavor layers (e.g.,

chocolate, peanut butter, strawberry), textural components (e.g. crisps, nuts, wafers), and nutritional bonuses (e.g. fiber, vitamins, minerals) (Loveday and others 2009). Protein is commonly blended with the additional components and is necessary to obtain the higher protein contents in some HPN bars (Banach 2012). The protein source (including blends and hydrolysates) will impact HPN bar texture, flavor, consumer acceptance, and stability (Childs and others 2007; Adams 2008).

Food bars are generally formulated to have water activity less than 0.65 (Loveday and others 2009) and some have water activity as low as 0.3 (Doherty and Ward 1997). Low water activity is needed to prevent microbial growth and ensure consumer safety since many HPN bars are not subjected to heat treatment (Liu and others 2009). The moisture content of HPN bars is typically 10% to 15% w/w (Zhu and Labuza 2010) and use low molecular weight humectants such as sorbitol, glucose, fructose, maltodextrin, glycerol, agave syrup, rice syrups and high-fructose corn syrup to control water activity. Along with the small amount of water present in the protein powders, carbohydrates syrups are usually the only source of water in HPN bars. Such syrups (containing ~70% solids) act to hold the bar ingredients together (Adams 2008). Sugar alcohols are used as low calorie sweeteners and for their humectant properties. Sorbitol and maltitol are two common sugar alcohols used in HPN bars. Glycerol, a polyol with a water activity-lowering effect, may also be incorporated into the HPN bar matrix (Liu and others 2009; Loveday and others 2009, 2010).

Fats make up about 10% to 15% of the HPN bar matrix (Zhu and Labuza 2010) although this may be higher for low carbohydrate bars. Fats used include vegetable shortening (McMahon and others 2009; Adams 2008), cocoa butter (Loveday and others

2009; Loveday and others 2010), canola or soy oil (Adams 2008), or even any type of food grade oil (Gautam and others 2006).

High-Protein Nutrition Bar Manufacture

During manufacturing, the dry ingredients such as protein powders are mixed with the carbohydrate syrups and the lipid material and then formed into a bar shape with no heat applied except for a possible chocolate coating. Immediately after manufacture most nutritional bars have a soft nougat-like texture and become harder during storage. There has been virtually no published research on the role of processing on HPN bar hardness even though this influences distribution of components throughout the bar (Aguilera and Stanley 1999; Aguilera 2000).

High-Protein Nutrition Bar Problems

In foods, texture has a significant effect on product acceptance by consumers because texture perception influences overall sensory appreciation (Wilkinson and others 2000; McMahon and others 2009). Hogenkamp and others (2011) reported that texture, not flavor, is the main determinant of satiety in dairy products. The formation of micro- and macrostructures in concentrated protein systems such as HPN bars is not fully understood (Purwanti 2012) and so hardening during storage is still a problem.

High-protein nutrition bars should maintain shelf stability for a minimum of six months if stored at room temperature (McMahon and others 2009) but stability for greater than 12 months is desired (Imtiaz and others 2012). The onset of hardening in HPN bars begins soon after production and bars can become hard, rigid, and difficult to chew within six months of storage at room temperature (Gautam and others 2006). Even

though the complexity of HPN bars make it difficult to pinpoint the actual causes of bar hardening, bar manufacturers have come up with numerous products that are designed to keep bars soft over time such as milk protein powders (Stefan 2003), high water activity peptide containing powders (Gautam and others 2006), and hydrolyzed protein containing powders (Gerdes 2005; Childs and others 2007; Labuza and others 2007; McMahon and others 2009).

Some companies have developed specialized MPC and WPC powders for use in HPN bars (Hutchinson 2009; Imtiaz and others 2012) to improve softening and cohesiveness when used as the sole protein and in combination with other proteins. Banach (2012) reported that low temperature extruded MPC extruded at 65°C and 120°C lessened textural change when compared with HPN bars formulated with unmodified MPC80.

Strategies for slowing bar hardening include use of plasticizers, hydrolyzed proteins or blended proteins instead of a single protein were found to lead to more stable product properties (Zhou and others 2008a, 2008b; Li and others 2008; Liu and others 2009; McMahon and others 2009; Loveday and others 2009, 2010). Using hydrolyzed proteins has drawbacks such as higher cost, bitter off-flavors and negative textural changes, and is also harder to use because if over-mixed the hydrolyzed proteins tend to lose their softening effect and tend to stick to equipment (McMahon and others 2009).

Whey protein prices have risen with the development of functional and protein fortified foods (Smithers 2008). Whey protein and soy protein are nutritionally comparable though the caseins in MPC are digested more slowly, allowing for nitrogen retention and muscle growth post-exercise (Tang and others 2009). Despite these

advantages, MPC performs poorly when incorporated into HPN bars because of having a crumbly texture lacking cohesiveness needed to hold the HPN bar together (Li and others 2008) as well as bar hardening. The mechanism of instability in HPN bars formulated with MPC has not been determined. Suggested mechanisms for hardening include moisture migration, limited intermediate water, phase separation, disulfide bond formation and protein aggregation (Loveday and others 2009, 2010; Li and others 2008; Zhou and others 2008a, 2008b; McMahon and others 2009). Proteins can form a strong network through intermolecular bonds (Labuza and others 2007; Zhou and others 2008a) and so inducing a non-homogenous distribution of the protein through processing could be a method to reduce long-range protein interactions. To find a solution to HPN bar hardening, more information is needed on the mechanisms underlying bar hardening.

Globular Proteins

Naturally occurring proteins may vary considerably in their molecular weights, but most globular proteins used as functional ingredients in the food industry fall in the 10- to 100-kDa molecular weight range (Damodaran 1996). In their native states, globular proteins have compact structures that are roughly spherical in shape; although the protein surface is normally highly irregular in topology and chemistry (Creighton 1993). The structures of globular proteins are highly dynamic, with the polypeptide chain and side groups fluctuating between many different conformations (Onuchic and others 1997; Freire 1998).

The conformation adopted may vary depending on pressure, temperature, solution composition, and depend on hydrophobic interactions, electrostatic interactions, hydrogen bonding, van der Waals forces, and configurational entropy (Dickinson and

McClements 1995; Damodaran 1996). Hydrophobic effects are a main driving force of formation and stabilization of compact structure of globular proteins (Tanford 1991). Protein use in foods is determined by their ability to bind other molecules, to undergo conformational changes, to self-associate, and to adsorb to interfaces (McClements 2002). However, detailed knowledge of three-dimensional structures of globular proteins in high-protein low water activity environmental conditions pertaining to HPN bars is limited, which makes interpretation of the molecular basis of protein functionality difficult. There are practical difficulties associated with characterizing the structure of proteins in complex systems. It is also important to recognize that food systems are rarely at thermodynamic equilibrium and consequently, proteins may be trapped in metastable conformations (McClements 2002). Protein functionality is influenced by size, shape, amino acid composition and sequence, net charge and distribution of charges, hydrophobicity/hydrophilicity ratio, secondary, tertiary, and quaternary structures, molecular flexibility/rigidity, and ability to interact/react with other components (Fennema 2008). The molecular characteristics of proteins are sensitive to small alterations in environmental conditions that change the balance of either the stabilizing and/or destabilizing forces. Many types of weakly interacting cosolvents present in foods are capable of altering protein functionality by modulating protein conformation, binding, self-association, and adsorption phenomena (McClements 2002).

Whey Proteins

Whey proteins are a main category of dairy proteins and contain several component proteins, including β-lactoglobulin (β-Lg) (48-58%), α-lactalbumin (α-La) (13-19%), glycomacropeptide (12-20%), immunoglobulins (8-12%), bovine serum

albumin (6%), lactoferrin (2%) and lactoperoxidase (0.5%) (Bonnaillie and Tomasula 2008). Commercial whey protein powders typically come in three major forms: WPC containing 35% to 85% (wt/wt) WPI containing ≥90% protein and hydrolyzed WPC (Lopes and others 2006). As noted by Wilcox and Swaisgood (2002), WPI processing steps include those for WPC with additional diafiltration to concentrate the proteins.

The α-La molecule in milk is a compact, low-molar mass (14.2 kDa) globular protein. The isoelectric point of α-La is 4.1 (Vasbinder 2002). It is more structurally stable than β-Lg because it has no free sulfhydryl groups, although it does contain four disulfide bonds (Farrel 1988). α-Lactalbumin is a calcium-binding protein (Hiraoka and others 1980), and removal of calcium results in profound conformational changes equivalent to those occurring through acid denaturation (Kronman and others 1981; Permyakov and others 1981). These changes include the irreversible unfolding of the molecule and a decrease in denaturation temperature of 20°C (Bernal and Jelen 1984). α-Lactalbumin has a denaturation temperature of 62°C making it the whey protein least resistant to unfolding when milk is heated (de Wit and Klarenbeek 1984) but is reversible upon cooling.

β-Lactoglobulin exists mainly as a dimer that consists of an ellipsoid measuring 6.45 nm by 3.6 nm. The β-Lg monomer has a molar mass of 18.3 kDa and comprises 162 amino acids (Vasbinder 2002). β-Lactoglobulin has a denaturation temperature of 78^{o}C (de Wit 1981). After dissociation, the monomers produced (Sawyer 1969; McKenzie 1971) unfold and then polymerize by sulfhydryl interchange followed by further aggregation (Harwalker 1980). Irreversible denaturation of β-Lg occurs above pH 7.5.

Today, whey proteins are in demand in the food industry, as they have been associated with perceptions of it being a nutritious protein, (Sharma and Shah 2010). They are widely used as ingredients in many food products and dietary supplements, such as body building protein powders, HPN bars, infant formula, high-protein drinks and energy drinks, extruded snacks, confectionary, and convenience foods products (Fox 2003).

However, during food processing and storage, whey proteins are liable to denaturation followed by aggregation (de Wit 1990; Bryant and McClements 2000; Havea and others 2002). The aggregation of proteins in a food matrix can result in dramatic changes in microstructure and texture. In dilute solutions, the denaturation of whey proteins causes the formation of soluble and insoluble aggregates (de Wit 1990; Havea and others 2002) through intermolecular disulfide bonding and noncovalent interactions (Bryant and McClements 2000) and will result in increased turbidity and precipitation in dairy beverages during processing and storage. At high-protein concentrations, protein networks can form whose structure and texture depend on composition and concentration of whey proteins as well as temperature, pressure, pH, and ionic strength (Boye and others 1995; Patel and others 2005). In general, whey proteins form either a particulate gel network or a fine-stranded (or filamentous) gel network. The particulate gels appear opaque and contain large aggregates (from several hundred to several thousand nanometers), while the fine-stranded gels are transparent and contain "flexible strands or more rigid fibrils" (Bryant and McClements 1998; Foegeding 2006).

When a protein such as α-La or β-Lg unfolds it requires energy and an endothermic peak occurs in the thermogram using differential scanning calorimetry

(DSC) (Bernal and Jelen 1985). The denaturation temperature (T_m) of the protein is determined from the position of the endothermic peak and is measured in the absence and presence of cosolvent. If an added material (cosolvent), such as a polyol in an aqueous system, increases the denaturation temperature then the cosolvent stabilizes the protein structure. If a cosolvent decreases the denaturation temperature the cosolvent destabilizes the protein structure (McClements 2002). By measuring the temperatures at which these changes occur, thermal stability and denaturation temperatures of proteins can be determined.

Bernal and Jelen (1985) demonstrated that whey protein T_m depends on pH, presence of milk sugars and the presence of fatty acids and is dominated by the β-Lg fraction. Paulsson and Dejmek (1990) found that β-casein had no effect on enthalpy, while α-casein lowered the T_m of all the whey proteins by 2 to 3°C. κ-Casein also lowered the denaturation temperature of β-Lg by 3°C. In a review of previous literature, Paulsson and Dejmek (1990) reported that in 10% to 20% protein concentrations in 0.1 M phosphate (pH 7.0) and heated at 5°C per minute, β-Lg had a T_m of 79°C, α-La had a T_m of 65°C and bovine serum albumin (BSA) had a T_m of 64°C. Boye and others (1997) reported that α-La has two reversible thermal transitions with T_m at 40°C for its other isoform. Anema (2000) reported that the T_m of β-Lg was dependent upon the protein concentrations and T_m increased at higher protein levels.

The presence of disulfide bonds plays an important role in thermal denaturation and T_m. In β-Lg there is one free thiol and two disulfide crosslinks and there are 4 in α-La that act as physical restraints for the freedom of motion after thermal denaturation

(Tanford 1968). The free thiol on β-Lg is capable of forming new disulfide bonds with other thiols and through interchange with existing disulfide bonds (de Wit and Klarenbeek 1981).

Above 40°C, β-Lg dimers begin to dissociate into monomers and these monomers are important during β-Lg heat denaturation. Heating to <70°C, there are changes in the structure of β-Lg that are reversible upon cooling while above 70°C denaturation of β-Lg becomes irreversible because of aggregation (de Wit and Swinkels 1980). When an aqueous cosolvent such as sorbitol is added, the thermal stability of β-Lg is increased such that the temperature at which denaturation occurs increases (Chanasattru and others 2008). de Wit and Klarenbeek (1981) observed an additional endothermic event of β-Lg that occurred between 130 and 150°C. However, this peak disappeared when 2-mercaptoethanol was added, suggesting that this endothermic event was due to the melting of residual protein conformations and perhaps the breakdown of disulfide bonds.

Milk Protein Concentrates

Milk protein concentrate is produced using ultrafiltration to remove the lactose from skim milk so that it contains a range of protein contents (42% to 85%) that maintain the same casein to whey protein ratio as skim milk. Moisture in MPC powders ranges from 3.5% to 5%, and lactose content is inversely related to protein content. There is no standard of identity for MPC in the United States (Mistry 2002; Gerdes 2008) and the United States Department of Agriculture first reported MPC production numbers in its 2009 report, which defined MPC as a dry milk powder containing 40.0% to 89.9% protein (USDA 2010). Milk protein concentrate powders are divided into three

categories: low-protein (≤ 55% protein), intermediate-protein (55% < protein < 80%), and high-protein (> 80% protein) (De Castro-Morel and Harper 2002). A milk protein powder that content ≥90% protein is called a MPI (Kelly 2011). There have been some other methods used for making similar product by dry blending a caseinate with WPC or by precipitating casein from skim milk, and then blending with whey proteins and then spraying the mixture (Kelly 2011).

Milk protein concentrates contribute to water binding, gelation, whipping, emulsification, browning, flavor enhancement, thickening, and nutrition when used in food applications (Baldwin and Pearce 2005) and can provide an uncluttered ingredient label (Chandan 1997). Despite being a nutritionally complete protein, a potential drawback of using MPC in food applications is its limited reconstitution and dissolution. Using MPC as the only protein source in high-protein nutrition bars has the problem that after manufacture, the bars become unpalatably crumbly, and during storage, they harden, especially on the surface, resulting in reduced shelf life.

Some attempts at improving MPC bar properties include modification of protein structure and bettering protein function by application of heat and shear (Banach 2012). Toasting and extrusion (shearing) partially unfold and denature the protein molecules, enabling interactions with other protein molecules. Although for bar hardening it may be important to have an inert protein with limited interactions with other bar components. Extrusion and toasting reduced protein solubility and in general also reduced surface hydrophobicity, and water holding capacity of MPC. Using toasted MPC increased the rate of bar hardening while using MPC extruded at 65°C lessened textural changes during storage (Banach 2012).

Carbohydrates as Cosolvents

The carbohydrates in HPN bars are added in the form of a syrup into which the protein powder and other ingredients are mixed. Such sugar or polyol syrups contain about 70% solids and 30% water. Thus, there are twice as many sugar/polyol molecules as water molecules and in this type of environment can be considered as cosolvents. Carbohydrates in the form of reducing sugars can also directly interact with proteins in HPN bars via Maillard browning reactions with protein amine groups.

Cosolvents in foods come in a wide variety of types, having different sizes, shapes, and chemical groups. Even so, they can be conveniently divided into four categories depending on whether their effect on protein transitions (such as unfolding) is neutral, favorable, unfavorable or combined (McClements 2002).

Neutral cosolvents are non-ionized and neither promote nor oppose a protein transition, (McClements 2002), although they can alter the conformation and functional performance of globular proteins in aqueous solution (Saunders and others 2000; Timasheff 2002a, 2002b). This ability to alter properties of globular proteins in solution depends on their molecular characteristics, e.g., number, size, shape and interactions (Parsegian and others 1995; Timasheff 1998, 2002a). An appropriate type and amount of cosolvent can modulate protein functionality in a specific way (Chanasattru and others 2007), improve conformational stability of proteins to environmental stresses (such as heating, cooling, high pressure treatment or dehydration), or to promote desirable conformational changes (Timasheff 1998; Saunders and others 2000; McClements 2002).

A stabilizing cosolvent is one that opposes a protein transition. At a molecular level a cosolvent may stabilize a protein through a variety of different mechanisms. Many simple sugars (e.g., sucrose, glucose, trehalose) and polyols (e.g., glycerol) are stabilizing cosolvents (Timasheff 1993, 1998; Ebel and others 2000). It is thought that sugars and polyols stabilize proteins through steric exclusion mechanisms (Xie and Timasheff 1997a, 1997b, 1997c). At room temperature, trehalose stabilizes proteins because it has a greater preferential exclusion from the denatured protein than the native protein (Xie and Timasheff 1997a, 1997b, 1997c). However, at higher temperatures trehalose stabilizes proteins because of a greater preferential accumulation by the native protein than by the denatured protein. Glycerol stabilizes many globular proteins presumably because glycerol molecules interact less favorably with hydrophobic groups on protein surfaces than water molecules (McClements 2002).

A destabilizing cosolvent is one that promotes a protein transition through a variety of different mechanisms (McClements 2002). Such weakly interacting cosolvents include urea and guanidine hydrochloride and these are frequently used as protein denaturants when added at relatively high concentrations (Timasheff 1998). Such destabilizing cosolvents preferentially bind to protein surfaces and thus favor the unfolded state of the protein because it has a larger surface area that can interact with the cosolvent.

However, for certain types of protein and environmental conditions, urea and guanidine hydrochloride may act as stabilizers (Timasheff 1998). Other cosolvents, such as sodium lactate may also be a stabilizing cosolvent under some conditions but be a

destabilizing cosolvent under other conditions based on temperature and cosolvent concentration (MacDonald and others 1996b).

Cosolvents can decrease the overall molar volume and adiabatic compressibility of the proteins because of the ability of the cosolvent to cause expulsion of water-containing voids and increased intra-molecular bonding within the protein interior (Almagor and others 1998; Taulier and Chalikian 2002; Chanasattru and others 2008), and increasing the osmotic stress acting on the proteins (Timasheff 2002a, 2002b). The specific partial volume of β-Lg molecules decreases and its compressibility increases when glycerol or sorbitol was added as a cosolvent (Chanasattru and others 2008) This was attributed to changes in the properties of the protein interior and solvation layer with sorbitol being more effective than glycerol at decreasing protein volume at 50% (wt/wt). Glycerol has some surface activity and preferentially accumulates around hydrophobic regions on protein surfaces.

According to McClements (2002), preferentially excluded cosolvents increase the T_m of globular proteins because there is a reduction in the surface area from which the cosolvent molecules are excluded. On the other hand, preferentially accumulated cosolvents lower T_m because they tend to favor the unfolded state over the folded state as there is an increase in the surface area to which the cosolvent molecules can bind. In general, sugars and glycerol increase the thermal stability of most globular proteins; however, the magnitude of the enhancement depends on cosolvent type, cosolvent concentration, protein type, and solution conditions (e.g., pH and ionic strength).

Cosolvents can favor or oppose protein self-association, depending on their preferential interactions with the aggregated and nonaggregated states of the protein

(McClements 2002). Preferentially excluded cosolvents tend to favor the aggregated state whereas accumulated cosolvents tend to favor the non-aggregated state based on surface area to which the cosolvent molecules can bind. This can be concentration dependent and sucrose decreases gelation rate of whey proteins at low concentrations (< 15%) because of its affect on viscosity, but at higher concentrations, it increases gelation rate as it is sterically excluded and increases attraction protein-protein interactions (Kulmyrzaev and others 2000b).

Concentration of cosolvents must also be considered when studying functional properties of proteins as these properties may only be manifest when the protein is fully dissolved in water (Damodaran 1996). Preferentially excluded cosolvents tend to favor the solid state over the dissolved state, because there is a reduction in the surface area from which the cosolvent molecules are excluded. Preferential interactions will depend on the precise nature of the changes in the surface area and surface chemistry of a globular protein (McClements 2002).

Globular proteins, such as whey proteins, have regions on their surface that can bind ligands depending on the characteristics of the molecules involved (Wyman and Gill 1990; Friere 1998). A protein may have single or multiple binding sites on its surface (McClements 2002) that may influence each other based on changes in conformation of a protein molecule upon ligand binding. Such binding may be either reversible or irreversible. A cosolvent may either favor or oppose the reaction depending on whether the cosolvent is preferentially excluded or preferentially accumulated, respectively. The influence of a cosolvent also depends on interactions of the cosolvent with the ligand, and

these can be different than the cosolvents interactions with the protein (McClements 2002).

A protein dispersed in an aqueous phase along with a lipid bulk phase will partition between the bulk phase and the interfacial region according to its concentration and surface activity (Adamson 1990) and can undergo conformational changes after adsorption to the interface (Dickinson 1992;). This can promote interactions between neighboring proteins (e.g., via hydrophobic or disulfide bonds). Cosolvents can influence these interfacial conformational changes because of their differing interactions with the folded and unfolded states of the adsorbed protein (McClements 2002).

Protein adsorption at interfaces is thermodynamically favored in the presence of sucrose, because the overall volume of the excluded regions is decreased after adsorption (Rodriguez-Nino and others 1997; Guzey and others 2001). Stabilization of the folded state of the protein by sucrose then results in less surface denaturation of the protein after adsorption, leading to a reduction in the number of reactive groups capable of forming protein-protein interactions (McClements 2002).

Protein solubility is determined by the relative magnitude of protein-protein interactions compared to interactions with solvent and cosolvent molecules (Damodaran 1996). Having more favorable protein-solution (solvent/cosolvent) interactions than protein-protein and solution-solution interactions, then the protein molecules prefer to be surrounded by solution rather than by each other and so the protein tends to be soluble. The magnitude of these various interactions depends on the molecular characteristics of the protein, environmental conditions, and solution composition. Usually, aqueous solubility of a protein decreases as its surface hydrophobicity increases and its net

electrical charge decreases (Damodaran 1996). Cosolvents can alter solubility of proteins by altering the balance of these interactions or by altering protein conformation (Arakawa and Timasheff 1985). Determining this can be complex as it depends on the type of protein and cosolvents, cosolvent concentration, pH, and temperature so that cosolvents may have different affects on protein solubility under different conditions. For example, sucrose increases water solubility of a variety of globular proteins near their isoelectric points (Antipova and Semenova 1997a, 1997b; Conti and others 1997) but decreases water solubility at other pH (Antipova and others 1999). Temperature also influences how cosolvents affect protein solubility (Antipova and others 1999). Weakly interacting cosolvents are often added to aqueous solutions of globular proteins to stabilize them against unfolding or aggregation induced by freezing, heating, mechanical stress, pressure treatment or dehydration treatments (McClements 2002). Sucrose, sorbitol and maltodextrin act as cryoprotectants for proteins at least in part because of their preferential steric exclusion from the protein surface.

A cosolvent that effectively protects proteins from denaturation during frozen storage is likely to protect them during heating. Although, cosolvents that favor the folded state over the unfolded state of globular proteins also tend to increase protein-protein interactions (Baier and McClements 2001). Cosolvents are often added to protein-containing materials prior to heating and drying to increase protein stability (Allison and others 1998, 1999; Murray and Liang 1999, 2000). Air drying of aqueous solutions of β-Lg in the presence of relatively high concentrations of sugars so as to retain more of the protein in its folded state improves subsequent foaming capacity (McClements 2002) presumably because there is less loss of native structure of the protein.

High pressure may induce the unfolding of globular proteins and cosolvents can increase protein stability during high-pressure treatment (Dumay and others 1994). For example, adding sucrose to β-Lg solutions reduces it denaturation after pressure treatment (450 MPa, 25°C for 15 min). Polyols such as glycerol and sorbitol are also effective at reducing high-pressure denaturation of proteins (Mozhaev and others 1996; Athes and Combes 1998; Ashie and others 1999).

Cosolvents can also alter gelation of globular proteins by (1) changing the temperature at which the protein unfolds, (2) altering the magnitude of the attractive and repulsive forces between protein molecules, and (3) by increasing viscosity of aqueous solutions, which decreases the rate of protein-protein encounters (McClements 2002). There are contradictory reports on the effects of preferentially excluded cosolvents on protein gel properties that can be explained based on the balance between the affects of cosolvents on protein unfolding and protein aggregation. It has been observed that nonreducing sugars can increase protein gel strength (Kulmyrzaev and others 2000a; Rich and Foegeding 2000) while others have reported decreases in gel strength (MacDonald and others 1996a; Carvajal and others 1999). McClements (2002) proposed that the gel strength increases if the proteins have time to unfold while decreased gel strength occurs when the proteins do not have time to unfold. Reducing sugars increase gel strength of whey protein gels through Maillard browning reactions leading to increased covalent cross-linking of the proteins (Hill and others 1992; Rich and Foegeding 2000).

Carbohydrates and Maillard Browning

Color changes of foods can occur because of reactions among food constituents that produce brown pigments (Friedman 1996; Francisco and others 2000). One of these is Maillard browning that is the consequence of a series of reactions of amines, amino acids, peptides, and proteins primarily with reducing sugars. Another cause of browning is caramelization of sugars as a result of heating. The manufacture of HPN bars does not involve any heating and so caramelization is unlikely to occur. Maillard browning reactions are accelerated by high temperatures but can also occur at room temperature during long storage times. There are other browning reactions such as the enzymatic that produces quinones from phenols but these are not applicable to HPN bars. Non-enzymatic browning reactions resulting from the interactions of amino acids or proteins with reducing sugars are considered either positive or negative processes, depending on the particular food. Maillard reaction can generate desired color, flavor, aroma and texture in some foods but unwanted off-flavor development, flavor loss, discoloration, and loss of protein nutritional value in other cases.

The non-enzymatic browning of foods is the consequence of a complex series of chemical reactions among different food components. Typically, it is considered to be produced by carbohydrate-protein reactions (the Maillard reaction) but other food components have been shown to play a similar role in browning (Francisco and others 2000). In general, the Maillard reaction involves glycosidation of amino acids or proteins that then leads to a myriad of subsequent reactions in which a complex mixture of compounds is obtained (Labuza and Baisier 1992; Deyl and Miksik 1997). In foods, Maillard reactions typically involve the common reducing sugars glucose, fructose,

maltose and lactose. The first step of the reaction is the formation of a N-substituted glycosylamine from an aldose (or ketose) reacting with a primary amino group of an amino acid, peptide, or protein. This glycosylamine can then undergo Amadori rearrangement reactions to yield a 1-amino-1-deoxy-2-ketoses. The next step of the reaction is the dehydration or fragmentation of the sugar, or the Strecker degradation to produce both amino and non-amino compounds. Finally, a condensation of the products formed in the previous step can occur either among them or with amino compounds to form brown pigments and polymers.

Lipids

Fats make up 10% or more of HPN bars (Zhu and Labuza 2010), with higher levels in low carbohydrate bars. Fats incorporated into HPN bar include vegetable shortening (McMahon and others 2009; Adams 2008), cocoa butter (Loveday and others 2009, 2010), canola and soy oils (Adams 2008), and essentially any type of food grade oil (Gautam and others 2006). Physical properties of the fats in systems such as margarines, fat spreads and shortenings are strongly influenced by their polymorphic properties in a crystalline state (Sato 2001). Factors that influence the network properties of fats include crystalline habit (crystal polymorphism), chemical composition, solid/liquid ratios and as well as mechanical and thermal history during manufacture and storage (Marangoni and Rousseau 1998).

The compositional profiles of common vegetable oils are dominated by five fatty acid: palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3). Cocoa butter, coconut, palm and tallow contain high amounts of saturated fatty acids, while corn, canola, safflower, soy, and sunflower oils are dominated by unsaturated fatty

acids. Until recently, shortening was almost always made of partially hydrogenated vegetable oil and these are being removed from the marketplace because of health concerns and newer products avoid trans-fats by using a mixture of non-hydrogenated oil and fully hydrogenated oil.

Modern shortening is a vegetable-based product that is just barely solid at room temperature. It can be used as an ingredient when a recipe needs a flavorless fat more solid oil than margarine. Cocoa butter, is an edible vegetable fat extracted from the cocoa bean and contains a high proportion of saturated fats, derived from palmitic and stearic acids (Liendo and others 1997) and could serve as an alternative to partially hydrogenated vegetable oil when solid fats are required in a food product (Anonymous, 2012). Cocoa butter has a melting point of 34 to 38 °C.

Lipids and Browning Reactions

Autoxidation of fats is the main cause of oxidative deterioration of lipids. This proceeds via typical free radical mechanisms and hydroperoxides are the initial products. They are relatively unstable and enter into numerous complex reactions involving substrate degradation and interaction, resulting in a myriad of compounds of various molecular weights, flavor thresholds, and biological significance (Frankel 1998; Berdeaux and others 1999). Lipid oxidation can lead to polymerization to produce brown oxypolymers (Khayat and Schwall 1983). Lipid oxidation products can react with amines, amino acids, and proteins (Stansby 1957; Gillatt and Rossell 1992; Pokorny 1998).

The role of lipids in non-enzymatic browning reactions is a consequence of this tendency to be oxidized. Polyunsaturated fatty acids are oxidized into hydroperoxides

that react with amino acids to form unsaturated imines. Similar reactions may proceed with free radicals formed by hydroperoxide decomposition. The reaction is accompanied by decarboxylation of the respective amino acid, i.e., it is analogous to Strecker degradation (Pokorny 1981; Pokorny and Kolakowaska 2002). Oxidized fatty acid hydroperoxides are further cleaved into unsaturated aldehydes, which, again, are active precursors of the browning reactions after condensation with amine groups (Ishh and others 1992; Gillatt and Rossell 1992). Unsaturated yellow imines formed in all these reactions are slowly transformed into brown macromolecular substances by subsequent polymerization and polycondensation reactions (Pokorny and Sakurai 2002).

The interaction between oxidized lipids and amino acids and proteins may imply either the formation of physical complexes between the oxidized lipids and the protein or the formation of various types of covalent bonds, in addition to the production of protein radicals (Gardner 1979). Protein polymerization produced by reaction with peroxy free radicals generated during lipid peroxidation can occur during non-enzymatic browning (Gardner 1979; Kikugawa and others 1990). Furthermore, when proteins are exposed to peroxidized lipids, the lipids can bind with the protein through hydrophobic association and hydrogen bonds, as well as covalent bonds (Fransisco and others 2000). Analogously to the Maillard reaction between reducing sugars and amino groups, oxidized-lipid reactions with protein are very diverse.

Water and Complex Mixtures

Functional and structural properties of proteins in moist foods are affected by their interaction with water via hydrogen bonding and hydrophobic interactions. Many functional properties of proteins, such as dispersibility, wettability, swelling, solubility,

thickening/viscosity, water-holding capacity, gelation, coagulation, emulsification, and foaming depend on water-protein interactions (Fennema 2008). In intermediate moisture foods, such HPN bars, the balance of protein-protein and protein-water interactions is critical to its storage properties. Water can bind to charged groups via ion-dipole interactions and nonpolar residues via dipole-induced dipole interactions and hydrophobic effects. Hydration of proteins is related to its amino acid composition and the hydration capacity is increased with the number of charged amino acid residues.

Hydration of Proteins

Hydration of proteins is influenced also by environmental factors, such as pH, ionic strength, temperature, type of salts, and protein conformation, with the least hydration occurring at the protein's isoelectric pH (Fennema 2008). At low concentrations, salts increase hydration of proteins while at high concentrations, more water molecules are interacting with the salt ions, so protein hydration decreases.

Hydration, or water-binding capacity, of proteins generally decreases with temperature, because of decreased hydrogen bonding, decreased hydration of ionic groups and increased hydrophobic effects. Denatured food proteins generally exhibit low solubility in water. Their water-binding capacities, however, are not drastically different from those in the native state. Thus, water-binding capacity cannot be used to predict the solubility characteristics of proteins. The solubility of a protein is dependent not only on water-binding capacity but also on other thermodynamic factors.

In food applications, the water-holding capacity of a protein is more important than the water-binding capacity (Fennema 2008). Water-holding capacity refers to the ability of the protein to imbibe water and retain it against gravitational force. This water

refers to the sum of the bound water, hydrodynamic water, and the physically entrapped water. The physically entrapped water contributes more to water-holding capacity than do the bound and hydrodynamic water. Protein solubility depends on hydrophilicity and hydrophobicity of the protein surface that contacts the surrounding water (and cosolvent) rather than the average hydrophobicity and charge frequency of the molecule as a whole. Since a majority of hydrophobic residues are buried in the interior of the protein, only those nonpolar groups that are on the surface affect the solubility.

Water and Browning Reactions

The rate of the initial stage of the Maillard reaction and the rate of brown pigment formation is dependent on the amount of free water available in the food (Reynolds 1963). Maximum browning occurs at a water activity of 0.6 to 0.8 (Wolfrom and Rooney 1953). Water is produced during the Maillard reaction, so that the reaction occurs less readily in foods with high water activity values, while, at low water activity, the mobility of reactants is limited, despite their presence at increased concentrations (Fennema 2008). Including glycerol in a food shifts the rate maximum to lower water activity levels while sorbitol seems to act as an inhibitor of browning at all water activity values, probably because of its higher viscosity. Even at low moisture browning can occur in the glycol phase.

Protein-Cosolvent-Water Interactions

There are two types of preferential interactions for protein-water-cosolvent combinations (McClements 2002). Steric exclusion comes about from differences in molecular dimensions of cosolvent and water molecules and extends from the protein

surface a distance approximately equal to the radius of the cosolvent. Cosolvent sugar/polyol molecules are usually larger than water molecules and so they are sterically excluded from the region surrounding each protein molecule while the water molecules can enter this region (McClements 2002). Differential interactions result from differences in strength of molecular interactions of water and cosolvent molecules with the protein surfaces. When cosolvent molecules are less strongly attracted to the protein surfaces, then water molecules will be preferentially accumulated in the narrow region surrounding the protein, and excluded if the cosolvent is more strongly attracted (Timasheff 1998 2002b; McClements 2002; Chanasattru and others 2008).

Cosolvents may also be excluded from surface crevices or interior cavities in a protein because of their relatively large size (Parsegian and others 1995). Such exclusion leads to a concentration gradient between the cosolvent-excluded region and the cosolvent-rich bulk aqueous phase and the subsequent osmotic stress induces alteration in protein conformation to close the crevice or cavity (McClements 2002).

Phase separated structures can be formed in the presence of incompatible biopolymers (such as proteins and polysaccharides) where interactions between the different polymers are repulsive and/or when the two types of polymers show varying affinity towards the solvent (Grinberg and Tolstoguzov 1997). By separating into a protein-rich phase and a polysaccharide-rich phase the system gains entropy (with a resultant decrease in free energy). Usually one of the biopolymer-rich phases forms a continuous phase with the other being dispersed throughout (Tolstoguzov 1998; Weinbreck 2004). McMahon and others (2009) suggested that a similar phase separation

occurs in HPN bars resulting in the formation of a protein-rich phase and a cosolvent rich phase.

Li and others (2008) reported that some HPN bars hardened rapidly when used with polyol syrups but not when used with sugar syrups depending on the protein powder used. Polyols and sugars of similar molecular size should produce similar steric exclusion effects and so differences in hardening suggest differences in differential interactions based on differences in strength of molecular interactions between the cosolvent and solvent molecules and the protein surface (Chanasattru and others 2008). Protein surfaces are heterogeneous and contain various functional groups of differing polarity, shape, and size that can interact differently with cosolvent and solvent molecules, depending on their molecular characteristics. This then influences preferential exclusion or accumulation of cosolvents in the local domain around the protein surface.

Glycerol can act as a plasticizer in HPN bar systems better than larger polyols (Liu and others 2009) because it is less excluded from the protein surface and presumably less excluded from hydrophobic regions on the protein surface (Chanasattru and others 2008). According to McClements (2001) glycerol acts less favorably with protein hydrophobic regions than water because in the presence of a nonpolar group water can still rearrange and form hydrogen bonds with other water molecules. In contrast, such rearrangement of glycerol requires that the hydrogen bonds between its alcohol groups be broken.

Excluding the cosolvent from the local domain requires increasing its concentration in the remaining bulk solution. This can be thermodynamically unfavorable, and so protein conformation may change to minimize the entire protein–

solvent/cosolvent interface (Liu and others 2009). In some cases, such as with glycerol, this stabilizes the protein against unfolding, but when the polyol can interact strongly with the protein and is preferentially accumulated in the local domain, such as with propylene glycol, protein aggregation can be induced. Similarly, while sorbitol more effectively stabilizes β-Lg in its native state than glycerol (because of a larger gain in free energy if the protein unfolds), sorbitol can promote self-association of β-Lg into large insoluble aggregates (Chanasattru and others 2008).

HPN Model Systems and Bars

High-protein nutrition bars harden during room temperature storage, and such hardening accelerates when the bars are stored at higher temperatures (e.g., 32 to 35°C). Texture of HPN bars can be affected by extrinsic factors such as temperature and humidity (Wilkinson and others 2000) and intrinsic factors such as the structure and properties of the ingredients in the bar and how those ingredients interact with each other (Aguilera and Stanley 1999). McMahon and others (2009) reported that mixing of protein powders with a sugar/polyol syrup and vegetable shortening produced a dough in which most of the protein powder particles were dispersed throughout the sugar/polyol syrup. There were also numerous small syrup droplets that contained no protein that were attributed to mechanical shearing during the mixing process when the doughs were being manufactured. Some of the protein powder particles also become covered with lipid that prevents their further dispersion.

All of these microstructural elements are indicative of material in which the three ingredients are dispersed together with some fat, sugar syrup, and protein still being in particulate form, as well as formation of a combined water-cosolvent-protein phase.

Typically, HPN bars with the same level of protein have similar initial hardness and microstructural appearance as the protein powder particles are simply dispersed throughout the bar matrix without the system having reached an equilibrated state between the proteins, the carbohydrate cosolvents, the lipids, and the water (McMahon and others 2009). There is insufficient water in HPN bars for the proteins to be fully hydrated. However, the sugars and polyols themselves can function as weakly interacting cosolvents (McClements 2002) in addition to the water and have a stabilizing effect on protein structure (Crowe and others 1987).

During the first week of storage the microstructure of the bars becomes less particulate in appearance with more continuous lipid and water/cosolvent phases (McMahon and others 2009). This change is thought to take place as water migrates from the higher water activity sugar/polyol syrup to the lower water activity protein powders (Li and others 2008). In bars that exhibited increased hardening during storage (i.e., those in which HWPI has not been included in the formulation), McMahon and others (2009) reported that there were large (200 to 1000 μm) water/cosolvent regions that were devoid of protein that formed after the first week of storage. While in bars that included the bar softening hydrolyzed WPI (HWPI) such structure did not occur until extended storage times or not at all.

When MPC powder is used as the protein source in HPN bars, a lack of cohesiveness and a crumbly texture has been observed (Li and others 2008; Banach 2012) and so the use of MPC in HPN bars has not been very common (Baldwin and Pearce 2005). Bars formulated with high concentrations of MPC harden quickly, become unpalatable, and have reduced product shelf life (Li and others 2008; Imtiaz and others

2012). Whey protein concentrates, WPI, HWPI, and soy protein powders have been used more frequently as they provide better functionality (Imtiaz and others 2012).

Browning of HPN Bars

High-protein nutrition bars develop a brown color during storage based upon the use of reducing sugars in the bar formulation, hydrolyzed proteins, and the temperature of storage (McMahon and others 2009). Using a sugar syrup containing glucose, fructose or other reducing sugars promotes Maillard browning reactions and will cause the bars to lose whiteness (the HPN bar doughs are initially white to cream colored). Such browning reactions are accelerated when HWPI is used in the bars because of the abundance of free amino groups produced upon hydrolysis of proteins. Using HWPI plus a sugar syrup containing reducing sugars can result in very high rates of browning and HPN bars that are almost black in appearance after 6 wk storage at 32°C (McMahon and others 2009). High-protein nutrition bars made using sorbitol and intact WPI remains white under the same storage conditions even though extensive bar hardening occurred.

Hardening Mechanisms in HPN Bars

Depending on their formulation, the onset of hardening in HPN bars can begin within days after bar manufacture and then become hard, rigid, and difficult to chew within six months of storage at room temperature (Guatam and others 2006, McMahon and others 2009). The mechanism leading to the hardening of protein bars has not been conclusively identified, although a number of hypotheses have been proposed. These include:

1. Moisture migration between the protein and water/cosolvent (Gallo-Torres 2002; Li and others 2008; Loveday and others 2009; Hogan and others 2012).
2. The physical state of water (Ruan and Chen 1998; Li and others 2008; Zhou and others 2008a, 2008b; McMahon and others 2009).
3. Protein aggregation (Labuza 2008; Zhou and others 2008b; Liu and others 2009).
4. Macroconstituent phase separation between the water, cosolvent and protein portions of the HPN bar matrix (McMahon and others 2009; Loveday and others 2010).
5. Maillard reactions between protein amino groups and reducing sugars when used in the bar formulation (Labuza and others 2007; Labuza 2008).
6. Shifts in the glass transition temperature of the sugars/poyols (Hartel 2001; Labuza 2008; Li and others 2008; Hutchinson 2009; Liu and others 2009; McMahon and others 2009).
7. Crystallization of sugars (Halliday 2005; Adams 2008; McMahon and others 2009).
8. Other interactions between proteins and minor components, such as Na^{+}, K^{+}, Ca^{2+}, or Mg^{2+} ions, leading to altered protein conformation and induced moisture migration (Book 2008).

To find a superior solution to the bar hardening problem, more information is needed on the mechanisms underlying bar hardening. An area that has not been well described is the interactions that can occur on the surface of protein in HPN bars after manufacture and during storage. It is still unclear which mechanism is primarily responsible for hardening and how to use milk proteins such as MPC in bar formulations.

CHAPTER 2.

HYPOTHESIS AND OBJECTIVES

I hypothesize that hardening of high-protein-high polyol food systems (such as HPN bars) during storage occurs because of weakly interacting surface interactions involving protein side-chains, water and the polyol cosolvent.

Efforts to explore this hypothesis focused on the following objectives:

Objective 1. Determine if previously reported protein-polyol phase separation occurs in HPN bars during storage.

Objective 2. Learn how changes to (a) protein (b) polyol, and (c) lipid influence texture and hardening of HPN bars.

Objective 3. Determine formulations to reduce hardening in HPN bars made using WPI and to improve texture of bars containing MPC.

CHAPTER 3.

EXPERIMENTAL DESIGN

This research was conducted in three phases. An initial experiment involved production of HPN bars using various proteins, polyols, and lipids with the intent of establishing a baseline for texture, color and some chemical aspects of HPN bars and their change during storage. Following this experiment, a series of small scale model systems were prepared to investigate a broad variety of formulations including use of some food additives not included in the first experiment to determine possible beneficial effects on reducing bar hardening. Finally, based on the outcomes of the model systems, another set of HPN bars were manufactured and tested.

Phase 1. Changes in Color, Hardness, State of Water and Protein, and Microstructure of HPN Bars based on Type of Protein, Carbohydrate and Lipid.

Testing For Protein Polyol Phase Separation. A reference bar was made consisting of 33.9% WPI, 46.7% sorbitol syrup and 19.4% vegetable shortening (Table 3-1) to verify that previously reported phase separation (McMahon and others 2009) occurs during storage and is related to bar hardening. This was designated as Bar 1 and was the reference HPN bar formulation. Packaged bars were at stored at 22 and 35°C to compare room temperature and accelerated storage, and sampled at d 0, 6, 21, 42, 70, 119, 175, 224 and d 0, 2, 8, 15, 29, 43, 60, and 90, respectively. Analysis included visual observation of the bars, water activity, state of water (bound, intermediate, bulk) and heat

Table 3-1 Percent composition of a reference bar (Bar 1) and other high-protein nutrition bars made during Phase 1 trials.

Bar	WPI[1]	MPC[2]	Sorbitol syrup[3]	Glycerol[4]	Vegetable Shortening	Cocoa Butter
1	33.9	0	46.7	0	19.4	0
2	0	33.9	46.7	0	19.4	0
3	33.9	0	0	46.7	19.4	0
4	33.9	0	46.7	0	0	19.4

[1]Whey protein isolate (90% protein, 4.5% moisture).
[2]Milk protein concentrate (80% protein, 5.0% moisture).
[3]Sorbitol syrup (70% solid sorbitol and 30% water).
[4]Glycerol 99.7%

denaturation of β-lactoglobulin and α-lactalbumin using DSC, color, hardness, and microstructure. Bars were made in duplicate on separate occasions.

Comparing the Influence of MPC versus WPI. Bars in which WPI was replaced with MPC were made at the same times as the reference bars, and stored and analyzed in the same manner. This formulation was designated as Bar 2 (Table 3-1).

Comparing the Influence of Glycerol versus Sorbitol. Bars in which sorbitol syrup was replaced with glycerol were made at the same times as the reference bars, and stored and analyzed in the same manner. This formulation was designated as Bar 3 (Table 3-1).

Comparing the Influence of Cocoa Butter versus Shortening. Bars in which shortening was replaced with cocoa butter were made at the same times as the reference bars, and stored and analyzed in the same manner. This formulation was designated as Bar 4 (Table 3-1).

Phase 2. An Exploration of how Combining Different Proteins, Polyols and Lipids, and other Additives in HPN Bar Formulations Influences Hardening.

Tween 20. Model systems were made using the same formulation as Bars 1, 2 and 3, with and without addition of the polysorban surfactant Tween 20 at a level of 1% (wt/wt) of polyol content. Samples were tested for hardness, water activity and color immediately after manufacture and after 43 d storage at 35°C. All samples were prepared in duplicate.

Calcium Chelation. Model systems consisting of MPC, sorbitol syrup and shortening as used for Bar 2 were made with and without addition 3% (wt/wt) of a 33% (wt/wt) disodium citrate solution. Samples were tested for hardness, water activity and color immediately after manufacture and after 43 d storage at 35°C. All samples were prepared in duplicate.

Protein Level. Model systems were made similar to Bar 1 (containing WPI) and Bar 2 (containing MPC) but with protein powder contents ranging from 31.4% to 37.1% (wt/wt) instead of the 33.9% used in Phase 1. The ratio of sorbitol syrup to shortening was kept constant. Samples were tested for hardness and color immediately after manufacture and after 29 d storage at 35°C. All samples were prepared in duplicate.

Sorbitol and Glycerol. Model systems were made using various ratios of sorbitol syrup and glycerol as the polyol component and compared to Bar 1 (WPI) and Bar 2 (MPC). Twenty percent, 50%, 80% and 100% of the sorbitol syrup was replaced with glycerol. Samples were tested for hardness, water activity and color immediately after manufacture and after 43 d storage at 35°C. All samples were prepared in triplicate.

Shortening and Cocoa Butter. Model systems were made using mixtures of shortening oil and cocoa butter at different ratios and compared to Bar 4 (containing cocoa butter). Samples were tested for hardness and color immediately after manufacture and after 43 d storage at 35°C. All samples were prepared in triplicate.

Lactose. Model systems were made based upon Bar 1 (WPI) but protein increased to 37.1% (with 44.5% sorbitol syrup and 18.4% shortening). To this formulation was added 1% or 5% (wt/wt) lactose. An additional model system was made in which part of the WPI was replaced with lactose yielding the formulation 28.9% WPI, 5% lactose, 46.8% sorbitol syrup 19.3% shortening). Samples were tested for hardness and color immediately after manufacture and after 29 d storage at 35°C. All samples were prepared in duplicate.

Oxidized Lipid. Model systems were made based upon Bar 1 (WPI, sorbitol syrup, and shortening) and Bar 2 (WPI, glycerol, and shortening) in which the shortening was replaced with soybean oil. Two samples of soybean bean oil were used, a freshly purchased oil and an oxidized oil (stored at 22°C for ~2 yr). Samples were tested for hardness and color immediately after manufacture and after 43 d storage at 35°C. All samples were prepared in duplicate.

Phase 3. Impact of Multi-Sized Cosolvents and Mixed Milk and Whey Proteins on Prevention of Protein Aggregation and Hardening of HPN Bars.

Testing For Protein Polyol Phase Separation. Bars were made consisting of 38.0% WPI, 44.0% sorbitol syrup and 18.0% vegetable shortening (Table 3-2) to verify that previously reported phase separation (McMahon and others 2009) occurs during

Table 3-2 Percent composition of high-protein nutrition bars during Phase 3 trials.

Bar	WPI[1]	MPC[2]	Sorbitol Syrup[3]	Glycerol[4]	Vegetable Shortening
1	38.0	0	44.0	0	18.0
2	19.0	19.0	44.0	0	18.0
3	38.0	0	35.2	8.8	18.0
4	19.0	19.0	35.2	8.8	18.0

[1]Whey protein isolate (90.0% protein, 4.5% moisture)
[2]Milk protein concentrate (80% Protein, 5.0% moisture).
[3]Sorbitol syrup (70% sorbitol, 30% water).
[4]Glycerol (99.7%).

storage and is related to bar hardening. This was designated as Bar A and was the control HPN bar formulation. Packaged bars (in water activity pan with cover, parafilm, and aluminum foil) were stored at 35°C (accelerated storage) and sampled at d 1, 7, 14, 21, 42, 63, and 84. Analysis included visual observation of the bars, water activity, state of water (bound, intermediate, bulk), denaturation of α-La and β-Lg using DSC, color, hardness, testing for disulfide bond formation by measuring free and total cysteine groups, solubility in 8 M urea and 5 mM dithiothreitol (DTT), and by SDS-PAGE, and microstructure by confocal laser scanning microscopy (CLSM). Bars were made in triplicate on separate occasions.

Comparing the Influence of WPI+MPC versus WPI. Bars in which WPI was added with MPC were made at the same times as the control bars, and stored and analyzed in the same manner. This formulation was designated as Bar B (Table 3.2).

Comparing the Influence of 80% sorbitol + 20% glycerol versus sorbitol. Bars in which sorbitol syrup was replaced with 80% sorbitol + 20% glycerol were made

at the same times as the control bars, and stored and analyzed in the same manner. This formulation was designated as Bar C (Table 3.2).

Comparing the Influence of 50% WPI + 50% MPC and 80 % Sorbitol + 20% Glycerol versus WPI + Sorbitol. Bars in which WPI and sorbitol was replaced with 50% WPI + 50% MPC and 80 % sorbitol + 20% glycerol were made at the same times as the control bars, and stored and analyzed in the same manner. This formulation was designated as Bar D (Table 3.2).

CHAPTER 4.

MATERIALS AND METHODS

Manufacture of HPN Bars

Bar Ingredients. Whey protein isolate (Provon 190, 4.5% moisture, 90% protein) was donated by Glanbia Nutritionals (Twin Falls, ID) and MPC (5% moisture, 80% protein) was donated by Idaho Milk Products, Inc. (Jerome, ID). Vegetable shortening (Crisco®) was from The J. M. Smucker Co. (Orrville, OH) and was made from soybean oil, fully hydrogenated cottonseed oil, partially hydrogenated cottonseed and soybean oils, and mono and diglycerides. Sorbitol syrup (30% moisture, 70% sorbitol) was from Archer Daniels Midland Company (Decatur, IL). Glycerol (99.7%) was from KIC Chemical Inc. (New Paltz, NY).

Phase 1. The method of McMahon and others (2009) was followed in which bars were made in 3-kg batches at Glanbia Nutritionals pilot plant (Twin Falls, ID) by mixing protein powder with polyol syrup and lipid until a smooth nougat-like texture was achieved (≤1 min). The formulations for the four HPN bars made in the phase 1 trials are shown in Table 3.1. The dough was formed into small cylindrical masses and fed into a bar former (Bepex Hutt, Leingarten, Germany) that rolled the dough out and cut it into about 75 bars (6.5 x 3 x 1 cm each). The finished bars were refrigerated for 30 min and then packaged individually into moisture-barrier foil-lined Mylar pouches (Sorbent Systems, Los Angeles, CA), heat sealed and labeled for storage.

Phase 2. Bars were made in small 33-g batches by mixing protein powder, polyol syrup, lipid and other ingredients as needed, using a spatula in small plastic cup until a smooth nougat-like texture was achieved (≤1.5 min). The mixture was transferred then to

plastic sample containers (4 cm diameter x 1 cm high; Decagon Devices, Inc., Pullman, WA), and a tight filling lid placed on the container and then the container and lid were sequentially wrapped in parafilm and aluminum foil to prevent moisture loss.

Phase 3. Bar doughs based on formulations shown in Table 3-2 were made in 1-kg batch by mixing protein powder with polyol syrup and lipid until a smooth nougat-like texture was achieved (≤1 min) using Bosch Universal Plus 800 W mixer (Bosch USA) at speed setting 2 using cookie dough paddles. The dough was pressed onto an aluminum pan (30 x 25 cm with a 1-cm lip) and a wooden roller used to form the dough into a 1-cm thick sheet. Then a plastic ring (4 cm diameter) was used to cut out ~65 circular portions of the dough, that were then placed into plastic sample containers (4 cm diameter x 1 cm high; Decagon Devices Inc.) and a tight filling lid placed on the container. The container and lid were sequentially wrapped in parafilm and aluminum foil to prevent moisture loss.

Bar Storage

Bars for phase 1 were stored at room temperature (22°C) and under accelerated storage at 35°C for up to 224 and 90 d, respectively. Bar samples were removed at required times, and tested on the same day or frozen at -80°C and kept frozen until analyzed. Model system during phase 2 were stored at 35°C for 29 or 43 d. Bars made in phase 3 were stored at 35°C for 84 d and samples removed at required times and tested within 1 d.

Water Activity

Water activity testing was performed using a Aqua Lab CX-2 meter (Decagon Devices Inc.) according to manufacturer's instructions and calibrated at water activity of 0.760 (using 6.0 molal NaCl) and 0.250 (using13.41 molal LiCl). Samples were measured by placing sufficient bar material to cover the entire bottom of the plastic sample cup (Decagon Devices, Inc.) and the cup inserted into the meter.

Differential Scanning Calorimetry

Bulk water and intermediate water content of HPN bars and protein denaturation transitions were measured using DSC (TA Instruments DSC model Q20 1963 with RCS cooling system, New Castle, DE). Five to ten milligrams of sample was taken from the center of the bars and placed into tared Tzero aluminum pans and lids (TA Instruments), then hermetically sealed and accurately weighed.

Phase 1 Trials. Samples were cooled to 10°C, held isothermally for 1 min, then heated to 120°C with a ramp of 5°C/min to analyze the endothermic denaturation of the proteins. An empty pan and lid were used as a reference. Sample pans were re-weighed after analysis to confirm hermetic sealing. TA Universal Analysis software was used to analyze the onset and peak denaturation temperatures and to determine denaturation enthalpy. Enthalpic change was then calculated on a protein basis according to (Zhou and others (2008b) as follows:

ΔH (J/g protein) = ΔH (J/g sample)/protein content of sample (g/g)

A second DSC analysis was performed to determine state of water in the sample. Samples were prepared as described above, then cooled rapidly to -40°C, held

isothermally for 5 min, heated to -24°C at 5 °C/min, and then further heated to 10 °C at 2 °C/min. TA Universal Analysis software was used to analyze the melting temperature and enthalpy related to melting of intermediate (-40°C < onset < 0°C) and bulk water (onset ~0°C). Based on the total moisture content of the sample, percent bulk water, intermediate water, and bound water (i.e., water not frozen at -40°C) were then calculated based on latent heat of fusion according to (Zhou and others 2008b) using 350 J/g water as calibrated using the DSC.

Phase 3 Trials. Bar samples during phase 3 trials were analyzed for both state of water and protein denaturation in a single run on 1, 21, 42, 63 and 84 d after manufacture. Samples were prepared as describe above, placed in the DSC, then cooled to -40°C, held isothermally for 10 min, then heated to 120°C at 5°C/min.

Protein

Protein content of bars was determined by micro-Kjeldahl (Rapid Kjeldahl System, Labconco, Kansas City, MO) according to AOAC Official Method 2001.11 using N x 6.38.

Color

Color analysis was performed using a Miniscan XE Plus portable colorimeter Model 45/O-S (Hunter Associates Laboratory Inc., Reston, VA) bench-top colorimeter operating with D65 northern daylight light according to Vissa and Cornforth (2006). Standardization was using black and white tiles covered with plastic wrap. Bar and model system samples were removed from their container and covered in plastic wrap. Color was expressed in terms of the CIELAB color space with the coordinates being L* (0-100,

estimation of lightness), a* (red-green) and b* (yellow-blue) (Pagliarini and others 1990). Mean color values were determined from five measurements taken at different spots on the sample.

In phase 3 trials, whiteness index (WI) based on Vargas and others (2008) in which 0 = black and 100 = white as well as color change during storage (ΔE) based on Banach (2012) were also calculated as follows:

$$WI = 100 - [(100 - L^*)^2 + (a^*)^2 + (b^*)^2]^{½}$$

$$\Delta E = [(a^* - a^*_0)^2 + (b^* - b^*_0)^2 + (L^* - L^*_0)^2]^{½}$$

in which L*, a* and b* are color values after storage and L^*_0, a^*_0 and b^*_0 are color values immediately after manufacture.

Hardness

Hardness was measured as maximum load during a 1 mm/s penetration test using a TA.XT Plus texture analyzer (Texture Technologies, Ramona, CA) with 5-kg load cell. During phase 1 trials a TA-42 45° chisel knife blade was used. When bar samples were made during phase 2 and phase 3 trials and placed into the 4-cm diameter plastic cups it was necessary to switch to using a TA-45 incisor blade (1-cm wide with flat end). Samples were tempered to room temperature (~22°C) for 2 h then tested using a crosshead speed of 1 mm/s and an activation load of 1 g-force. Three measurements for hardness were collected on different parts of the sample. Hardness was measured as the peak load (g-force) during penetration of the sample to 10 mm (phase 1 and 2 trials) and to avoid any bottoming out of the penetration probe this was reduced to 7 mm for phase 3

trials. Relative changes (%RC) was calculated according to Liu and others (2009) as follows:

$$\%RC = [(H - H_0) / H_0] \times 100$$

where H is the hardness after storage and H_0 is hardness on day of manufacture.

Bar Microstructure

Microstructure of bar samples was examined using a CLSM (LSM 710, Carl Zeiss Microscopy LLC, Thornwood, NY) equipped with a Kr/Ar laser. Slices approximately 8 x 8 x 2 mm were cut from bars at room temperature (~22°C) and placed on a microscope slide. One drop of a 0.02% (wt/wt) fluorescein isothiocyanate (FITC) (Sigma-Aldrich Inc., Saint Louis, MO) solution in absolute acetone was applied to the sample and allowed 60 s to penetrate. One drop of a 0.02% (wt/wt) Nile Red (Sigma-Aldrich, Inc) solution in absolute acetone was then applied and allowed to penetrate for 60 s. Samples were then inverted and placed stained side down on a glass bottom culture dish (35-mm petri dish, 10-mm microwell; MatTek Corporation, Ashland, MA) and placed into the holder of the microscope. Images were collected using 10x and 20x objective lens from two locations for each sample.

Extent of Disulfide Bonding

Extent of disulfide bonds in bar samples were evaluated by (1) determining protein solubility in the presence and absence of dithiothreitol (DTT), (2) by the difference between the level of free cysteine sulfhydryl groups compared to total sulfhydryl groups, (3) manufacturing bars with DTT included in the formulation, and (4) the presence or absence of protein aggregates in polyacrylamide gel electrophoresis.

Solubility. Five gram (±0.0005) of sample was suspended in 50 ml of four different solvent systems: (1) water, (2) 5 mM DTT, (3) 8 M urea, and (4) 8 M urea plus 5 mM DTT. The mixtures were stirred for 45 min (120 oscillations/min) at 45°C by magnetic stirrer then centrifuged at 13,600 x *g* (Model RB-5, Sorvall) at 10°C for 20 min and visually examined for sedimented material.

Sulfhydryl groups and disulfide bonds. Free and total sulfhydryl groups were determined and disulfide bonds calculated according to Thannhauser and others (1987) and Chan and Wasserman (1993) with some modification. The principle of the method is to suspend the sample in a buffer containing urea and then react it with a color reagent that simultaneously reacts with both soluble and insoluble protein with the release of a soluble chromophore. Ellman's reagent 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was used for determination of free sulfhydryl groups while disodium 2-nitro-5-thiosulfobenzoate ($NTSB^{2-}$) which hydrolyzes disulfide bonds was used for the determination of total sulfhydryl groups. The $NTSB^{2-}$ solution was synthesized according to the method of Thannhauser and others (1987) with minor modifications: 0.1 g DTNB was dissolved in 10 ml 1 M $NaSO_3$. The pH of the mixture was adjusted to 7.5, and 50 µl of a 0.1 M ammoniacal solution of $CuSO_4$ (25 ml 0.8 M NH_4Cl mixed with 25 ml 0.2 M $CuSO_4$) was added. Reaction with DTNB or $NTSB^{2-}$ released the soluble 2-nitro-5-thiobenzoate anion (NTB^{2-}), which has an extension coefficient of 13,600 $M^{-1}cm^{-1}$ at 412 nm.

Free sulfhydryl group content was determined by suspending 1.5 g of sample (~0.5 mg protein) in 10 ml of reaction buffer consisting of 8 M urea, 3 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS), and 0.2 M

Tris-HCl, pH 8.0 and the mixture stirred for 45 min (120 oscillations/min) at 45°C. One milliliter of the suspension was then diluted with 9 ml of the reaction buffer and 20 µl of 10 mM DTNB, pH 8.0 was added. The reaction mixture was stored for 15 min in the absence of light at room temperature (~22°C) then centrifuged at 13,600 *g* for 20 min at 10°C to remove particulate matter. Absorbance of the supernatant was measured at 412 nm against a reagent blank. The concentration (C) of free sulfhydryl thiol groups in µM/g protein was calculated from the molar absorbance as follows:

$$C = A/13{,}600 \times D$$

where A is absorbance and D is the dilution factor based on the amount of protein in the original sample.

Total sulfhydryl thiol content was determined by suspending 1.5 mg of sample in 10 mL of reaction buffer consisting of 8 M urea, 0.1 M sodium sulfite, 3 mM EDTA, 1% SDS, 0.2 M Tris-HCl, pH 9.5 and stirred for 45 min at 45°C. One milliliter of the suspension was diluted with 9 ml of the same buffer and 20 µl of 10 mM $NTSB^{2-}$, pH 9.5 was added. The reaction mixture was incubated in absence of light for 25 min at room temperature (~22°C) and centrifuged at13, 600 *g* for 20 min at 10°C to remove particulate matter. Absorbance was read at 412 nm and concentration of total sulfhydryl thiol groups calculated using the above equation. Disulfide bond content was calculated as half the difference between total thiol group content and free thiol group content.

Bar manufacture. Bars were made in which 0.017% and 0.075% of a 5 mM DTT solution was added into the formulation, and the bars stored at 35°C for 43 d and then tested for hardness and compared to bars made without DTT. All sample were prepared in triplicate.

Gel electrophoresis. A 0.5-g sample of bar (0.15 to 0.16 g protein) was suspended in 10 ml of 8 M urea solution and stirred for 45 min (120 oscillations/min) at 45°C. Then 1 ml of the suspension was diluted with 9 ml of running buffer (25 mM Tris-Cl, 192 mM glycine and 0.1% SDS, pH 8.3), then washed using ~5 ml n-hexane and shaking for ~2 min to remove lipid and the upper hexane layer was removed. Forty microliter of defatted sample suspension protein was mixed with 20 µl of loading buffer (150 mM Tros-Cl. pH 6.8, 6% SDS, 0.3% bromophenol blue, 1% β-mercaptoethanol, 30% glycerol) in small plastic vials. Then 25 to 30 µl was loaded into the sample wells of the polyacrylamide gel (Tris-Glycine Gel, 4% to 20% gradient, 1.5 mm thick; No. 4-20-D-1.5-HMC10P; Jule Inc., Milford, CT) with two gels run simultaneously at 140 volt for 50 min. The gel was stained with 0.25% Coomassie Blue Brilliant R250 in 25% methanol, 67.5% water and 7.5% glacial acetic acid) for 10 to 15 min and destained with 7.5% acetic acid plus 25% methanol and 67.5% water. The molecular weight of each protein band was checked with known protein standard. Standards were prepared using WPI and MPC and included on each gel as well as a molecular weight standard (SigmaMarker MW 6.5 to 200 kDa). Presumptive identification of bands was based on the WPI and MPC standards and according to Loveday and others (2009).

Statistical Analysis

Phase 1. A randomized block split-split-plot design was used with main-plot factors being bar formulation (Bars 1 to 4), the split-plot factor was storage temperature (22°C and 35°C) and the split-split-plot factor was designated equivalent storage times for room temperature and accelerated storage (see Appendix A for program code). The experiment was duplicated. Random factors: replicate (2) and replicate*treatment. Fixed

factor: treatment (4), temperature (2), and storage time (8). Data were analyzed in SAS 9, 3 (SAS Inst. Inc. Cary, NC) with a significance level of a $P \leq 0.05$ using the Proc GLIMMIX procedure. Differences of lsmeans was used to determine significant differences.

Phase 2. Model bar samples were made in duplicate or triplicate and means and standard deviation calculated.

Phase 3. A randomized block 2 x 2 factorial split-plot design was used with main-plot factors being protein (2 treatments) and carbohydrate (2 treatments). Storage time at 35°C was the split-plot factor (see Appendix B for program code). Random factors: replicate (3), replicate*treatment. Fixed factor: treatment (4), temperature (1), and storage (7). Within each treatment, the bars were stored at 35°C and each bar was measure at 7 storage times. Data were analyzed in SAS 9, 3 (SAS Inst. Inc.) with a significance level of a $P \leq 0.05$ using the Proc GLIMMIX procedure. Differences of lsmeans was used to determine significant differences.

CHAPTER 5.

PHASE 1: CHANGES IN COLOR, HARDNESS, STATE OF WATER AND PROTEIN, AND MICROSTRUCTURE OF HPN BARS BASED ON TYPE OF PROTEIN, CARBOHYDRATE AND LIPID.

INTRODUCTION

Four HPN bars were manufactured in which bars made using MPC, glycerol and cocoa butter were compared to a reference bar made using WPI, sorbitol syrup and shortening. The formulation of the reference bar was based on previous work (Adams 2008; McMahon an others 2009) in which bar hardening was proposed to result from a phase separation between the protein and sorbitol. It had also been shown that bar hardening during storage can be reduced by inclusion of a partially hydrolyzed WPI into the bar formulation. This however, has the disadvantage that it promotes Maillard browning of the bars because of the increased number of available amino groups that can react with reducing sugars. Such browning is accelerated when a carbohydrate such as high fructose corn syrup is used as the solvent/cosolvent mix rather than a polyol such as sorbitol syrup.

This experiment was designed to (1) test the previously proposed hypothesis that a carbohydrate-protein phase separation occurs during storage, and (2) to provide a reference platform for further investigation of HPN bar formulations using combinations of ingredients. The basis for the proposed phase separation that then promotes protein aggregation, was the observation using CLSM of HPN bars that the large (black) areas in

the micrographs lacked fluorescence from either Nile Red or FITC, represented areas that contained the polyol syrup but were depleted of protein.

RESULTS

Bar Manufacture

During manufacture, differences were observed in the texture and firmness of the dough (and the initial bars) depending on the ingredients used. The reference dough (Bar 1) containing WPI, sorbitol syrup and shortening mixed readily within ~1 min producing a soft malleable dough that extruded well and was easily formed into bars with nougat-like consistency as reported by Adams (2008) and Childs and others (2007). The dough formed using the Bar 2 containing MPC had a more crumbly texture and was whiter in appearance than Bar 1. When glycerol was used as the carbohydrate (Bar 3), the dough mixed together readily since glycerol is less viscous than sorbitol syrup but it was necessary to cool the dough as it was soft and sticky and could not be extruded at room temperature. In contrast, the Bar 4 dough made using cocoa butter was firmer than the others and required a longer time for the ingredients to be blended into a uniform dough.

Visual Observations During Storage

In general, the bars were initially white to cream in color, with Bar 2 containing MPC being visually the whitest probably because of light scattering related to the protein being mainly in the form of colloid-size casein micelles. During storage all bars also underwent changes in color. Bar 2 containing MPC, remained the whitest through 90 d storage at 35°C (Figure 5.1) and during 224 d storage at 22°C. Bar 1 was initially very

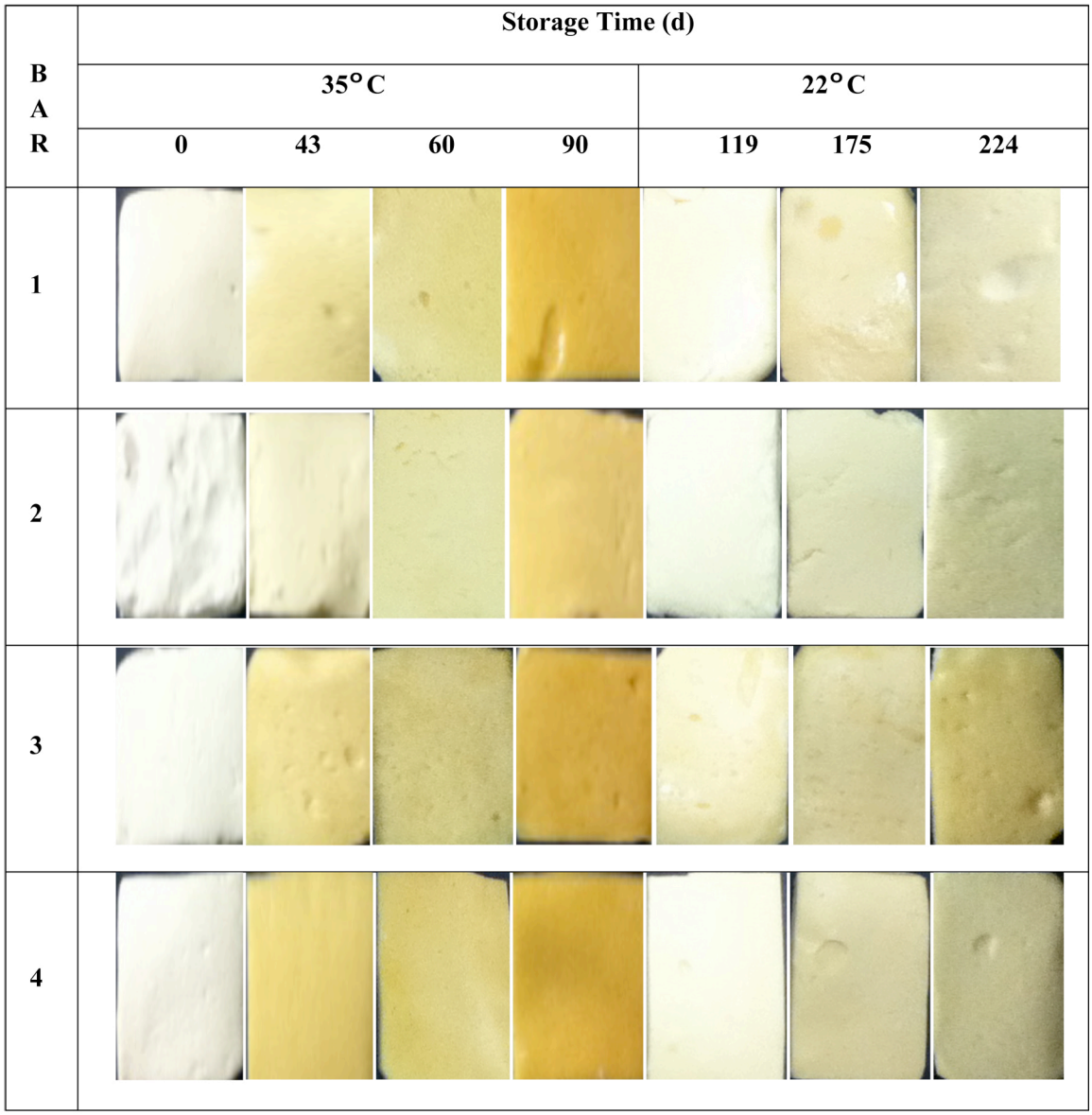

Figure 5.1 Change in color of high-protein nutrition bars during storage at 22 and 35°C as formulated in Table 3.1 in which Bar 1 was made using whey protein isolate, sorbitol syrup and shortening, in Bar 2 whey protein isolate was replaced with milk protein concentrate, in Bar 3 sorbitol syrup was replaced with glycerol, and in Bar 4 shortening was replaced with cocoa butter.

white but brown coloration was observed within 7 d when stored at 35°C, as was also the case with Bar 3. The intensity of brown coloration increased throughout storage and progressed quicker at 35°C than 22°C, as was expected for Maillard browning reactions. All of these bars were formulated using polyols (sorbitol or glycerol) rather than reducing sugars, and so the extent of browning was much lower than occurred with bars formulated with high fructose corn syrup (McMahon and others 2009). There was also less browning as no hydrolyzed WPI was used in this study. Based on visual observation, the initial extent of brown coloration in the four bars was Bar 1 > Bar 4 > Bar 3 > Bar 2 while, after 2 d storage at 35°C and through 84 d it was Bar 3 > Bar 4 > Bar 1 > Bar 2. Further information on color changes based on measurement of L*, a* and b* measurements is presented subsequently.

Consistency of the bars also changed during storage. Based on visual observation and feel of the bars by hand, the reference bar (Bar 1) had the best consistency and remained flexible and taffy-like throughout storage. With Bar 2, the surface of the bar hardened quickly while the interior of the bar became crumbly during storage such that these bars lacked cohesiveness and were too crumbly. Bar 3 became very hard during storage with a rigid texture. The consistency of Bar 4 was similar to Bar 1 although slighter firmer.

During storage it became more apparent that there had been air included in the dough during mixing and the number and size of air voids in all the bars increased during storage. There were less air voids but of larger size in Bar 2 compared to Bar 1. In Bar 3 there was a greater number of smaller air voids, and these increased in size along with

Bar 3 becoming very hard during storage. Bar 4 also had a more homogeneous texture than Bar 1 and had less air voids that were more circular in shape.

Hardness

Both bar formulation and storage time significantly influenced bar hardness (Table 5.1). Since it is well known that bars harden faster at elevated temperature (McMahon and others 2009), an estimate was made at the beginning of this experiment on the relative rate of hardening that would occur during accelerated storage at 35°C compared to storage at 22°C. Consequently, bars were collected for analysis from storage at seven time points with 0, 6, 21, 42, 70, 119, 175, 224 d of 22°C storage being paired for statistical analysis 0, 2, 8, 15, 29, 43, 60, and 90 d at 35°C.

Table 5.1 ANOVA of hardness of high-protein nutrition bars[1] during storage[2] at 22°C and 35°C.

Type III Tests of Fixed Effects				
Effect	**Numerator DF**	**Denominator DF**	**F Value**	***P* > F**
Bar (B)	3	190	7741.92	<0.0001
Temperature (T)	1	1	0.14	0.7694
B*T	3	190	42.61	<0.0001
Storage Time (S)	7	190	3405.19	<0.0001
B*S	21	190	890.87	<0.0001
T*S	7	190	1.43	0.1953
B*T*S	21	190	3.64	<0.0001

[1]Bars were formulated as shown in Table 3.1.
[2]Bars were stored at both 22°C and 35°C and storage times of 0, 6, 21, 42, 70, 119, 175, 224 d at 22°C were considered comparable to 0, 2, 8, 15, 29, 43, 60, and 90 d storage at 35°C and paired accordingly for statistical analysis.

After manufacture (d 0), the bars were significantly different in hardness with peak force values of 297, 145, 71 and 574 g-force for bars 1, 2, 3 and 4 respectively (Table 5.2). Within 6 d of storage at 22°C and 2 d at 35°C, significant differences in hardness between Bars 1, 3, and 4 were observed compare with d 0, while an increase in hardness in Bar 2 was not observed until 21 d at 22°C and 8 d at 35°C. These differences in hardness between the bars persisted throughout storage as shown in Figure 5.2. Although the hardness values were only slightly different between Bar 1 and Bar 2, there was a difference in texture as the bars made using MPC (Bar 2) were much more brittle and tended to fracture during the hardness test. In contrast, the load during the penetration test for Bar 1 continued to increase with depth of penetration and no fracturing of the bars occurred.

When comparing hardness of bars during storage at 35°C to those at 22°C, our original prediction on comparable times during accelerated storage had been slightly underestimated, and there were some differences based on bar formulation. Forty-three days storage at 35°C produced the same level of hardness as storage at 22°C for 70 d for Bar 1, 119 d for Bar 2, 224 d for Bar 3 and 70 d for Bar 4. Storage for 90 d at 35°C was equivalent to 175 d at 22°C for Bars 1 and 4, 224 d for Bar 2 and >224 d for Bar 3. In general, while replacing shortening with cocoa butter increased the initial firmness of the bar, it had no effect on extent of hardening during accelerated storage. Using MPC instead of WPI, or glycerol instead of sorbitol syrup caused the hardening to be accelerated more at 35°C, showing a greater dependence of the hardening reactions with temperature in these bars.

Table 5.2 Change in Hardness of a reference[1] high-protein nutrition bar compared to bars in which whey protein isolate (WPI) was replaced with milk protein concentrate (MPC), sorbitol syrup was replaced with glycerol, or shortening was replaced with cocoa butter, during storage at 22 and 35°C.

Storage Time (d)	Hardness (g-force)			
	Bar 1 Reference	Bar 2 MPC	Bar 3 Glycerol	Bar 4 Cocoa Butter
22°C				
0	297^{B}	145^{D}	71^{E}	574^{p}
6	352^{yz}	153^{D}	329^{A}	791^{mn}
21	374^{wxy}	296^{B}	1123^{g}	826^{klm}
42	383^{wx}	323^{A}	1460^{e}	847^{jkl}
70	394^{vw}	424^{tu}	1557^{e}	873^{ijkl}
119	411^{uv}	465^{rs}	1664^{d}	885^{ij}
175	452^{st}	514^{q}	1755^{cd}	910^{i}
224	514^{q}	671^{o}	1836^{c}	1037^{h}
35°C				
0	297^{B}	145^{D}	71^{E}	574^{p}
2	334^{zA}	152^{D}	392^{vw}	749^{n}
8	366^{xy}	234^{C}	1267^{f}	791^{mn}
15	372^{wxy}	337^{zA}	1525^{e}	822^{lm}
29	377^{wx}	423^{tu}	1792^{c}	839^{jklm}
43	387^{vwx}	458^{rs}	1819^{c}	872^{ijkl}
60	427^{tu}	489^{qr}	2026^{b}	882^{ijk}
90	457^{s}	651^{o}	2232^{a}	923^{i}

[1]33.9% WPI, 46.7% sorbitol syrup, 19.4% vegetable shortening.

$^{abc\ldots xyzABCDE}$means with same letter were not significantly different, $\alpha = 0.05$.

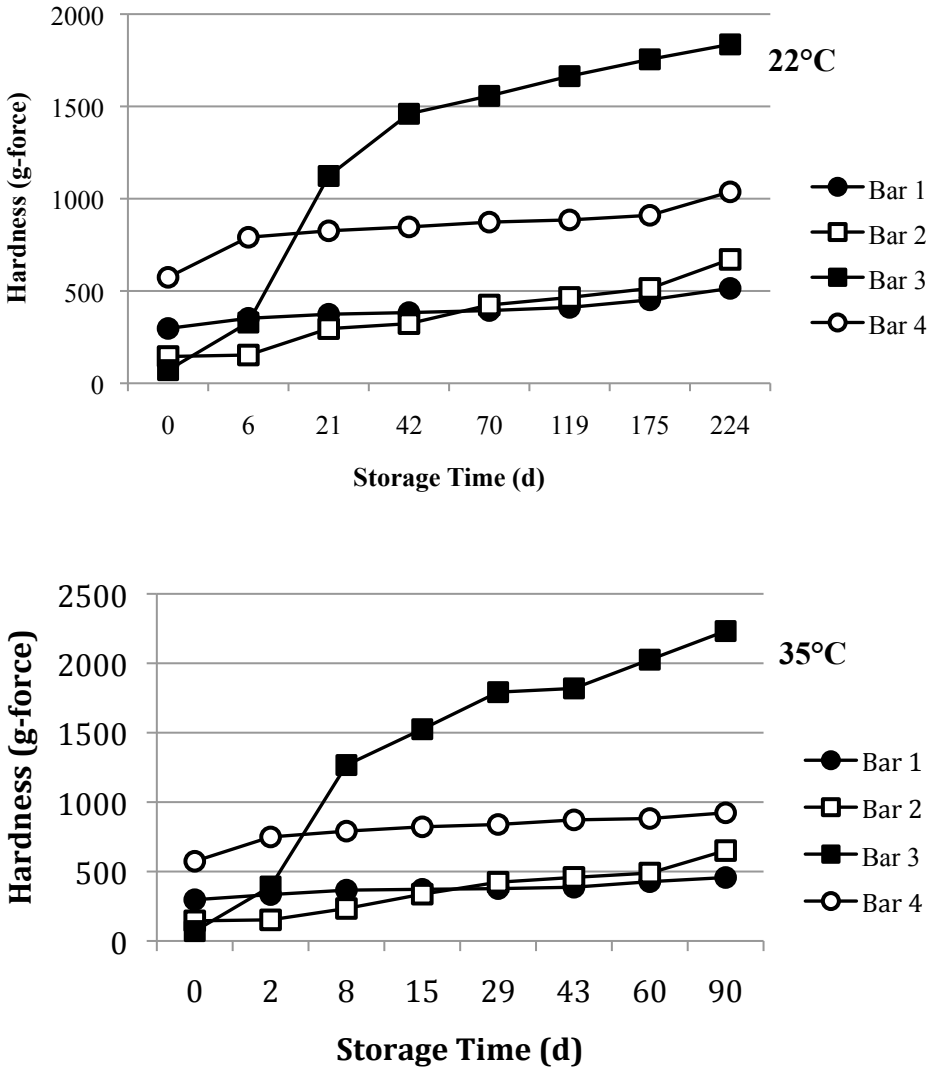

Figure 5.2 Change in hardness of high-protein nutrition bars during storage at 22 and 35°C as formulated in Table 3.1 in which Bar 1 was made using whey protein isolate, sorbitol syrup and shortening, in Bar 2 whey protein isolate was replaced with milk protein concentrate, in Bar 3 sorbitol syrup was replaced with glycerol, and in Bar 4 shortening was replaced with cocoa butter. See Table 5.2 for statistical differences.

While using glycerol instead of sorbitol syrup produced the softest dough and bar initially, it had the fastest rate of hardening during storage. Within 8 d of storage at 35°C and 21 d at 22°C, the hardness of Bar 3 far exceeded that of Bar 1 and was even harder than Bar 4 made using a lipid with a higher solid:liquid ratio (cocoa butter). At these storage times the hardness was Bar 3 > Bar 4 > Bar 1 > Bar 2, and after 224 d storage at 22°C the hardness was Bar 3 >> Bar 4 >> Bar 2 > Bar 1 with values of 1,836, 1,037, 671 and 514 g-force respectively.

Color

L*. All effects significantly influenced L* values (lightness) of the HPN bars (Table 5.3). Having a significant temperature effect indicates that color changes are more influenced by storage temperature than hardness and the bars stored at 35°C were noticeably darker in color than those stored at 22°C (Figure 5.1). As shown by McMahon and others (2009), the extent of browning has little if any influence on bar hardness. The extent of browning that occurred in this study was much less than observed by McMahon and others (2009) when high fructose corn syrup was used with hydrolyzed WPI. Those bars remained softer than a reference bar made using WPI and sorbitol syrup, even though they were almost black in color.

After manufacture (d 1), there were slight but significant ($P < 0.05$) differences in lightness of the bars as observed visually. The L* values for the bars were Bar 2 = Bar 3 > Bar 4 > Bar 1 (Table 5.4). During storage, all bars decreased in lightness with the greatest change occurring in Bar 3 that was made using glycerol. Bar 1 and Bar 2 were similar in lightness at most storage times, showing little influence of cocoa butter (compared to shortening) on browning. This was expected since Maillard browning

Table 5.3 ANOVA of L* color of high-protein nutrition bars[1] during storage[2] at 22°C and 35°C.

Type III Tests of Fixed Effects				
Effect	**Numerator DF**	**Denominator DF**	**F Value**	***P* > F**
Bar (B)	3	190	1946.78	<0.0001
Temperature (T)	1	1	377.41	0.0327
B*T	3	190	34.69	<0.0001
Storage time (S)	7	190	1721.20	<0.0001
B*S	21	190	70.72	<0.0001
T*S	7	190	89.77	<0.0001
B*T*S	21	190	11.99	<0.0001

[1]Bars were formulated as shown in Table 3.1.

[2]Bars were stored at both 22°C and 35°C and storage times of 0, 6, 21, 42, 70, 119, 175, 224 d at 22°C were considered comparable to 0, 2, 8, 15, 29, 43, 60, and 90 d storage at 35°C and paired accordingly for statistical analysis.

Table 5.4 Change in color- L* values of a reference[1] high-protein nutrition bar compared to bars in which whey protein isolate (WPI) was replaced with milk protein concentrate (MPC), sorbitol syrup was replaced with glycerol, or shortening was replaced with cocoa butter, during storage at 22 and 35°C.

Storage Time (d)	L*			
	Bar 1 Reference	Bar 2 MPC	Bar 3 Glycerol	Bar 4 Cocoa Butter
22°C				
0	87.5^{ef}	92.3^{a}	91.9^{ab}	90.2^{cd}
6	85.2^{i}	92.1^{a}	82.1^{lm}	86.9^{fg}
21	83.5^{j}	91.7^{ab}	81.2^{mnop}	83.3^{jk}
42	82.1^{lm}	91.2^{abc}	77.2^{vw}	82.3^{kl}
70	81.2^{mnop}	88.4^{e}	76.8^{wx}	80.7^{nopq}
119	80.5^{opq}	86.2^{ghi}	71.8^{bc}	80.0^{qr}
175	79.9^{qr}	82.0^{lm}	69.6^{EF}	79.2^{rs}
224	79.1^{rst}	81.3^{lmno}	68.4^{G}	78.7^{stu}
35°C				
0	87.5^{ef}	92.3^{a}	91.9^{ab}	90.2^{cd}
2	85.7^{ih}	91.6^{ab}	88.1^{e}	86.7^{fgh}
8	81.7^{lmn}	90.9^{bc}	75.4^{yz}	80.2^{pqr}
15	79.3^{rs}	89.8^{d}	75.3^{yz}	78.0^{tuv}
29	77.9^{uvw}	87.4^{ef}	71.9^{B}	77.1^{vwx}
43	76.1^{xy}	83.8^{j}	70.6^{ED}	74.8^{z}
60	73.5^{A}	79.6^{qrs}	70.0^{EDF}	73.3^{A}
90	70.8^{CD}	73.7^{A}	64.2^{H}	69.0^{FG}

[1]33.9% WPI, 46.7% sorbitol syrup, 19.4% vegetable shortening.

$^{abc\ldots xyzABCDEFG}$ means with same letter were not significantly different, $\alpha = 0.05$.

reactions involve non-lipid components of the bars. After 224 d of storage at 22°C, L* values for the bars were Bar 2 > Bar 1 = Bar 4 >> Bar 3. Browning occurred much more rapidly at 35°C, with equivalent storage times to 224 d at 22°C being 15 d for Bars 1 and 4, ~50 d for Bar 2, and ~70 d for Bar 3.

a*. Similarly to L* values, all effects (bar formulation, storage temperature and time) significantly influenced a* values of the HPN bars (Table 5.5). Initially all the bars had negative a* values, meaning they had a slight green tinge. During storage, a* values increased for all bars, although at different rates. The time for a* values to become positive and for a red tinge to develop in the bars stored at 22°C, was 42 d for Bar 1, 70 d for Bar 3, 119 d for Bar 2 and 224 d for Bar 4 (Table 5.6). Of these, only Bar 1 had an a*

Table 5.5 ANOVA of a* color of high-protein nutrition bars[1] during storage[2] at 22°C and 35°C.

Type III Tests of Fixed Effects				
Effect	**Numerator DF**	**Denominator DF**	**F Value**	***P* > F**
Bar (B)	3	190	848.58	<0.0001
Temperature (T)	1	1	5380.44	0.0087
B*T	3	190	9.51	<0.0001
Storage time (S)	7	190	6416.59	<0.0001
B*S	21	190	41.84	<0.0001
T*S	7	190	1602.03	<0.0001
B*T*S	21	190	18.67	<0.0001

[1]Bars were formulated as shown in Table 3.1.
[2]Bars were stored at both 22°C and 35°C and storage times of 0, 6, 21, 42, 70, 119, 175, 224 d at 22°C were considered comparable to 0, 2, 8, 15, 29, 43, 60, and 90 d storage at 35°C and paired accordingly for statistical analysis

Table 5.6 Change in color a* values of a reference[1] high-protein nutrition bar compared to bars in which whey protein isolate (WPI) was replaced with milk protein concentrate (MPC), sorbitol syrup was replaced with glycerol, or shortening was replaced with cocoa butter, during storage at 22 and 35°C

Storage Time (d)	a*			
	Bar 1 Reference	Bar 2 MPC	Bar 3 Glycerol	Bar 4 Cocoa Butter
22°C				
0	-0.72^{ABC}	-0.78^{CD}	-0.89 ^{EF}	-0.94^{F}
6	-0.66^{AB}	-0.70^{ABC}	-0.81^{DE}	-0.86^{DEF}
21	-0.30^{uv}	-0.54^{yz}	-0.52^{xy}	-0.62^{zA}
42	0.12^{q}	-0.37^{vw}	-0.23^{tu}	-0.52^{y}
70	0.60^{m}	-0.14^{rst}	0.22^{qp}	-0.28^{uv}
119	0.87^{l}	0.34^{on}	0.24^{op}	-0.20^{stu}
175	1.17^{k}	0.58^{m}	0.23^{op}	-0.16 ^{rst}
224	1.24^{k}	0.61^{m}	0.37^{n}	0.16^{qp}
35°C				
0	-0.72^{ABC}	-0.78^{CD}	-0.89^{EF}	-0.94^{F}
2	-0.51^{xy}	-0.70^{ABC}	-0.67^{AB}	-0.85^{DEF}
8	-0.08^{r}	-0.56^{yz}	-0.24^{tu}	-0.63^{zA}
15	0.26^{op}	-0.31^{uv}	-0.12^{sr}	-0.42^{xw}
29	1.51^{j}	0.66^{m}	0.54^{m}	0.27^{onp}
43	2.61^{f}	1.74^{i}	1.96^{h}	1.15^{k}
60	3.53^{d}	3.56^{d}	3.11^{e}	2.30^{g}
90	4.76^{b}	6.09^{a}	4.32^{c}	4.28^{c}

[1]33.9% WPI, 46.7% sorbitol syrup, 19.4% vegetable shortening.
$^{abc\ldots xyzABCDEF}$means with same letter were not significantly different, $\alpha = 0.05$.

value ≥1.0 by d 224. Increase of browning was accelerated when the bars were stored at 35°C, with positive a* values reached in 15 d for Bar 1 and in 29 d for the other bars. By 43 d storage at 35°C, all the bars had higher a* values than after storage at 22°C for 224 d, with Bar 1 > Bar 3 > Bar 2 > Bar 4. With further storage to 90 d at 35°C, a* values were Bar 2 > Bar 1 > Bar3 = Bar 4, with values of 6.09, 4.76, 4.32 and 4.28, respectively. So, even though Bar 2 made with MPC was initially the whitest, with extended storage at 35°C it had the most red color.

b*. Similar changes were observed in b* values of the HPN bars although the bar x temperature interaction was not significant (Table 5.7). Initially all bars had a slight

Table 5.7 ANOVA of b* color of high-protein nutrition bars[1] during storage[2] at 22°C and 35°C.

Type III Tests of Fixed Effects				
Effect	**Numerator DF**	**Denominator DF**	**F Value**	***P* > F**
Bar (B)	3	190	224.89	<0.0001
Temperature (T)	1	1	2273.97	0.0133
B*T	3	190	1.91	0.1291
Storage time (S)	7	190	1488.48	<0.0001
B*S	21	190	21.92	<0.0001
T*S	7	190	378.56	<0.0001
B*T*S	21	190	13.06	<0.0001

[1]Bars were formulated as shown in Table 3.1.
[2]Bars were stored at both 22°C and 35°C and storage times of 0, 6, 21, 42, 70, 119, 175, 224 d at 22°C were considered comparable to 0, 2, 8, 15, 29, 43, 60, and 90 d storage at 35°C and paired accordingly for statistical analysis.

yellow coloration, with b* values for Bar 4 > Bar 2 > Bar 3 = Bar 1 with values of 13.4, 11.7, 10.4 and 10.1, respectively (Table 5.8). During storage at 22°C, all bars increased in b* value, and after 224 d storage at 22°C the extent of yellowness was 15.3, 16.7, 17.3 and 18.0 for Bars 1, 2, 3 and 4, respectively. When stored at 35°C, the bars reached equivalent b* values after 29 d. Browning continued with storage at 35°C, with Bar 2 containing MPC having significantly more yellow color (b* = 32) than the other three bars (b* = 26).

Water Activity

Both bar formulation and storage time significantly influenced the water activity of the HPN bars. The interaction between bar formulation and storage time was also significant (Table 5.9). Initially the water activity of Bar 1 and Bar 4 were not significantly different (0.687 and 0.685 respectively), which was expected as they had identical non-lipid components. This similarity continued throughout storage at 35°C, although when stored at 22°C the water activity of Bar 1 increased slightly more ($P <$ 0.05) than Bar 4 (Table 5.10). The initial water activity of Bar 2 (0.701) was slightly higher than Bar 1 and Bar 3. This confirmed the slightly higher moisture content in MPC that increased overall bar water activity even though the MPC contained more lactose.

Bar 3 made with glycerol instead of sorbitol syrup had a much lower initial water activity (0.119). This was expected since the glycerol was 99.7% pure, whereas the sorbitol syrup contains 30% water. Water activity of Bar 3 increased to 0.164 after 21 d storage at 22°C, with no significant change upon further storage.

Table 5.8 Change in color b* values of a reference[1] high-protein nutrition bar compared to bars in which whey protein isolate (WPI) was replaced with milk protein concentrate (MPC), sorbitol syrup was replaced with glycerol, or shortening was replaced with cocoa butter, during storage at 22 and 35°C.

Storage Time (d)	b*			
	Bar 1 Reference	Bar 2 MPC	Bar 3 Glycerol	Bar 4 Cocoa Butter
22°C				
0	10.1^{J}	11.7^{DEFG}	10.4^{IJ}	13.4^{xyz}
6	10.7^{IJ}	12.3^{BCD}	11.8^{CDEF}	13.7^{wxy}
21	11.5^{EFGH}	12.2^{BCDE}	11.1^{FGHI}	14.9^{pqrst}
42	12.3^{BCD}	13.2^{yzA}	11.6^{DEFG}	15.0^{pqrs}
70	14.1^{tuvwx}	14.5^{rstuv}	15.0^{pqrs}	16.1^{lmn}
119	14.8^{qrstu}	15.2^{opqr}	14.7^{qrstu}	16.7^{jkl}
175	14.9^{pqrst}	15.6^{mnop}	16.3^{klm}	17.3^{ij}
224	15.3^{opqr}	16.7^{jkl}	17.3^{ij}	18.0^{ih}
35°C				
0	10.1^{J}	11.7^{DEFG}	10.4^{IJ}	13.4^{xyz}
2	10.7^{HIJ}	12.1^{BCDE}	11.0^{GHI}	13.7^{vwxy}
8	12.6^{ABC}	12.7^{zAB}	11.2^{FGHI}	15.3^{nopq}
15	14.0^{uvwx}	14.3^{stuvw}	12.7^{zAB}	17.1^{jk}
29	15.9^{lmno}	16.5^{jkl}	17.2^{j}	18.4^{h}
43	20.4^{f}	19.4^{g}	22.5^{d}	21.5^{e}
60	22.9^{d}	21.6^{e}	24.3^{c}	25.4^{b}
90	25.4^{b}	31.9^{a}	26.0^{b}	25.5^{b}

[1]33.9% WPI, 46.7% sorbitol syrup, 19.4% vegetable shortening.

$^{abc\ldots xyzABCDEF}$means with same letter were not significantly different, $\alpha = 0.05$.

Table 5.9 ANOVA of water activity of high-protein nutrition bars[1] during storage[2] at 22°C and 35°C.

Type III Tests of Fixed Effects				
Effect	**Numerator DF**	**Denominator DF**	**F Value**	***P* > F**
Bar (B)	3	190	494180	<0.0001
Temperature (T)	1	1	10.70	0.1889
B*T	3	190	2.51	0.0604
Storage time (S)	7	190	149.52	<0.0001
B*S	21	190	47.75	<0.0001
T*S	7	190	1.25	0.2770
B*T*S	21	190	1.58	0.0564

[1]Bars were formulated as shown in Table 3.1.

[2]Bars were stored at both 22°C and 35°C and storage times of 0, 6, 21, 42, 70, 119, 175, 224 d at 22°C were considered comparable to 0, 2, 8, 15, 29, 43, 60, and 90 d storage at 35°C and paired accordingly for statistical analysis.

Table 5.10 Change in water activity of a reference[1] high-protein nutrition bar compared to bars in which whey protein isolate (WPI) was replaced with milk protein concentrate (MPC), sorbitol syrup was replaced with glycerol, or shortening was replaced with cocoa butter, during storage at 22 and 35° C

Storage Time (d)	Water Activity			
	Bar 1 Reference	Bar 2 MPC	Bar 3 Glycerol	Bar 4 Cocoa Butter
22° C				
0	0.687^{qr}	0.701^{fghi}	0.119^{z}	0.685^{r}
6	0.693^{klmnop}	0.704^{abcdef}	0.139^{y}	0.690^{opq}
21	0.695^{klm}	0.706^{abcd}	0.164^{uv}	0.691^{mnopq}
42	0.697^{ijk}	0.705^{abcde}	0.167^{stu}	0.693^{klmno}
70	0.696^{jkl}	0.707^{a}	0.167^{stuv}	0.691^{mnopq}
119	0.699^{ghij}	0.707^{a}	0.168^{stu}	0.695^{klmn}
175	0.701^{edfg}	0.707^{ab}	0.167^{stu}	0.694^{klmno}
224	0.701^{efgh}	$0.704^{abcdefg}$	0.165^{tuv}	0.695^{jklm}
35° C				
0	0.687^{qr}	0.701^{fghi}	0.119^{z}	0.685^{r}
2	0.688^{pqr}	0.701^{defg}	0.144^{x}	0.687^{qr}
8	0.691^{mnopq}	0.702^{cdefg}	0.156^{w}	0.691^{mnopq}
15	0.695^{klm}	$0.702^{abcdefg}$	0.167^{stuv}	0.690^{nopq}
29	0.695^{jklm}	$0.703^{abcdefg}$	0.163^{v}	0.692^{lmnop}
43	0.697^{ijk}	0.705^{abcde}	0.167^{stu}	0.693^{klmno}
60	0.697^{hijk}	0.706^{abc}	0.170^{s}	0.694^{klmn}
90	0.695^{klmn}	0.704^{abcdef}	0.169^{st}	0.693^{klmno}

[1]33.9% WPI, 46.7% sorbitol syrup, 19.4% vegetable shortening.
$^{abc\ldots xyz}$means with same letter were not significantly different, $\alpha = 0.05$.

State of Water

Both bar formulation and storage time significantly influenced the distribution of water between bulk, intermediate and bound states (Table 5.11, see also Appendix A Tables A.1 to A.3). Storage temperature was not a significant effect, indicating that changes in state of water were accelerated at 35°C compared to 22°C.

A typical thermogram for analyzing the state of water in the HPN bars is shown in Figure 5.3 (thermograms for water, glycerol and sorbitol syrup are shown in Appendix A, Figures A.1 to A3). Bars 1, 2 and 4 contain 15% to 16% moisture, yet as seen in the thermogram, there is very little water that is frozen upon cooling to -40°C and subsequently observed to melt as the sample is warmed to 10°C. In the thermogram there

Table 5.11 ANOVA of bulk, intermediate and bound water of high-protein nutrition bars[1] during storage[2] at 22°C and 35°C.

Type III Tests of Fixed Effects					
Effect	**Numerator DF**	**Denominator DF**	**Bulk Water *P* > F**	**Intermediate Water *P* > F**	**Bound Water *P* > F**
Bar (B)	3	190	<0.0001	<0.0001	<0.0001
Temperature (T)	1	1	0.3227	0.0910	0.1405
B*T	3	190	0.2408	<0.0001	<0.0001
Storage time (S)	7	190	<0.0001	<0.0001	<0.0001
B*S	21	190	<0.0001	<0.0001	<0.0001
T*S	7	190	0.4459	<0.0001	<0.0001
B*T*S	21	190	0.7958	<0.0001	<0.0001

[1]Bars were formulated as shown in Table 3.1.
[2]Bars were stored at both 22°C and 35°C and storage times of 0, 6, 21, 42, 70, 119, 175, 224 d at 22°C were considered comparable to 0, 2, 8, 15, 29, 43, 60, and 90 d storage at 35°C and paired accordingly for statistical analysis.

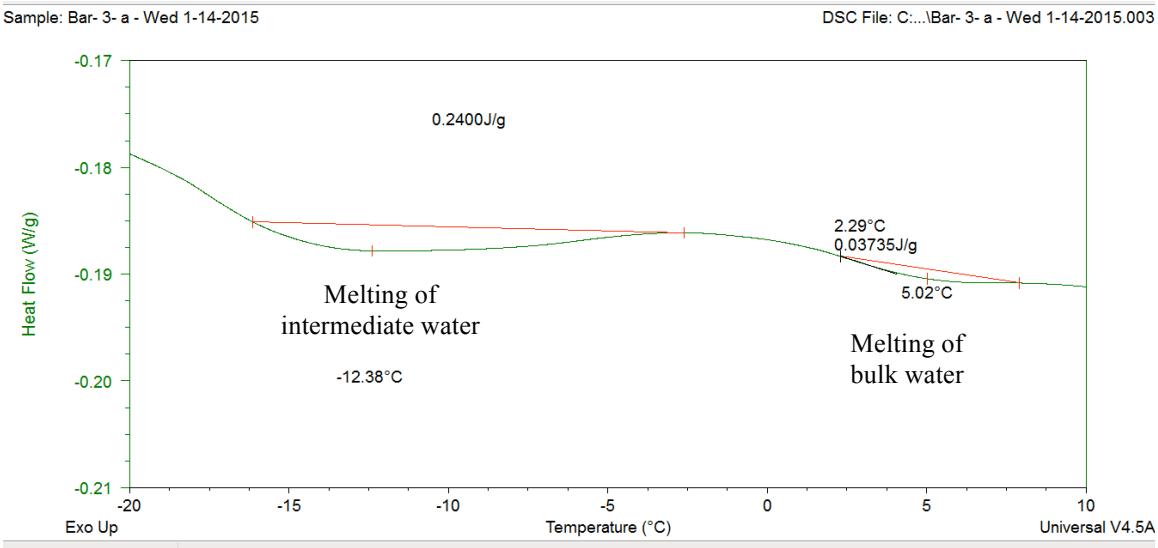

Figure 5.3 Example of a differential scanning calorimeter thermogram of high-protein nutrition bars used for determining extent of intermediate and bulk water endothermic transitions (exo up).

is a step as the heating rate is slowed from 5°C/min to 2°C/min at -24°C, but no endothermic transitions representing melting of ice into intermediate water was observed at temperatures <-24°C. A slight endothermic event was observed with onset temperatures between -20 and -5°C (see Appendix A, Tables A.4 and A.5) that was labeled as peak 1, and designated as melting of intermediate water.

A second small peak was observed as the sample was heated through the melting point of bulk water. It had onset temperatures between -2°C and 1°C, with peak temperature between -1°C and 5°C. This was because of a lag in response as the sample heated at 2°C/min through the melting point of water. This was labeled as peak 2 and was indicative of the presence of bulk water. Using the peak areas as a measure of ΔH,

enthalpy changes in J/g were then calculated. Peak 1 and peak 2 were then compared to the total amount of water, and the weight of the bars.

There was also only a small amount of intermediate or weakly interacting water (0.12 to 0.17 g/100 g solids) in the bars (Table 5.12, also see Appendix A, Table A.4 and A.5). All of the bars contained very little (≤0.02 g/100 g solids) bulk water (Table 5.12, also see Appendix A, Tables A.6 and A.7). Most of the water was strongly interacting and bound to other components. For bars 1, 2 and 4 the level of bound water was 18.2 to 18.4 g/100 g solids, while for Bar 3 (made using glycerol) the bound water level was only 1.4 g/100 g solids (Table 5.12, also see Appendix A, Tables A.6 and A.7). So, in the bars made using sorbitol syrup, ~99% of the water was so strongly interacting with proteins, sorbitol and other non-lipid solids, that it was not freezable at -40°C, with about 0.9% being intermediate water and only 0.1% existing as bulk water.

Intermediate water. Even though the amount of intermediate water in the bars was small (<0.1% of total water), there were significant differences between bars manufactured using sorbitol syrup (Bars 1, 2 and 4) with levels of 0.174, 0.167, 0.162 g/100 g solids (Table 5.12, see also Appendix A, Tables A.4 to A.7). There were small, but statistically significant, changes in state of the water during storage that followed the same trends for storage at 22°C and 35°C. After ~40 d of storage, there was a significant decrease in intermediate water of ~0.01 g/100 g solids (a loss of ~5% of the intermediate water) and this decrease continued though storage. By 224 d at 22°C and 90 d at 35°C, the decrease in intermediate water was ~10%. During the same period, there was a significant lowering of the onset temperature of the intermediate enthalpic transition

Table 5.12 Initial levels of bulk and intermediate water of a reference[1] high-protein nutrition bar compared to bars in which whey protein isolate was replaced with milk protein concentrate (MPC), sorbitol syrup was replaced with glycerol, or shortening was replaced with cocoa butter.

Bar	Bulk water (g/100 g Solids)	Intermediate water (g/100 g Solids)	Bound water (g/100 g Solids)
1. Reference	0.0193[a]	0.1742[a]	18.22[b]
2. MPC	0.0185[a]	0.1674[b]	18.47[a]
3. Glycerol	0.0145[b]	0.1206[d]	1.42[d]
4. Cocoa Butter	0.0182[a]	0.1620[c]	18.23[b]

[1]33.9% WPI, 46.7% sorbitol syrup, 19.4% vegetable shortening.
[abcd]means in the same column with same letter were not significantly different, $\alpha = 0.05$ based upon Table A.4 to A.7.

from about –19°C to about –11°C after about 60 d (see Appendix A, Tables A.4, A.5, A.9 and A.10). With further storage, the onset temperature for the intermediate peak again increased to about -19°C. This suggests that a portion of the intermediate water was becoming more strongly interacting with other bar components during the first portion of storage. Then as those interactions further increased, the water became sufficiently bound so that it was no longer freezable at -40°C. Then the remaining intermediate water had the same level of interactions as in the initial bars and, hence, the same onset melting temperature.

When glycerol was used as the solvent during bar manufacture (Bar 3), there was still some distribution of water between bulk, weakly interaction and strongly interacting states. About 10% of the water in Bar 3 was intermediate water, and accounted for 0.12 g/100 g solids. This was about 0.05 g/100 g solids lower than in the sorbitol bars (Table 5.12). During storage, the level of intermediate water decreased throughout the entire storage period, and dropped to 0.08 g/100 g solids after 224 d at 22°C and 0.07 g/100 g solids after just 60 d at 35°C.

Bulk water. Both bar formulation and storage time significantly influenced the bulk water of the bars (Table 5.11 and Appendix A, Table A.11). For the sorbitol bars (Bars 1, 2 and 4), there were small but significant differences in bulk water content with initial values of 0.0193, 0.0185 and 0.0182 g/100 g solids, respectively (Table 5.12). By about 70 d at 22°C and 15 to 43 d at 35°C, there was a 10% to 15% increase in bulk water (increase of ~0.003 g/100 g solids) (Appendix A, Tables A.6 and A.7). This continued throughout storage, with a larger increase occurring at 35°C. After 90 d at 35°C, there was an overall increase of ~25% of bulk water. Bars 1, 2 and 4 had bulk water contents of 0.0244, 0.0223, and 0.0238 g/100 g solids, respectively. Bar 3 (made using glycerol) had lower levels of bulk water but followed the same trend during storage. Initially these bars containing 0.0145 g/100 g solids of bulk water, and then increased to 0.0172 g/ 100 g solids after 90 d at 35°C (Appendix A, Table A.7).

Bound water. As with bulk and intermediate water, the level of bound water was influenced by both bar formulation and storage time (Table 5.11). The level of bound water was Bar 2 > Bar 4 > Bar 1 >> Bar 3, with values of 18.47, 18.22, 18.23, and 1.42 g/100 g solids, respectively (Table 5.12 and Appendix A Table A.6). Levels of bound water increased during storage, with a larger increase occurring at 35°C than at 22°C. After 224 d storage at 22°C, bound water levels between the bars still had the same relationship with Bar 2 > Bar 4 > Bar 1 >> Bar 3, with levels of 18.49, 18.26, 18.24 and 1.46 g/100 g solids, respectively. While after 90 d storage at 35°, the bound water levels were 18.50, 18.29, 18.24 and 1.47 g/100 g solids, respectively for Bars 2, 4, 1 and 3 (Appendix A, Table A.7).

Protein Denaturation

An example of a DSC thermogram showing denaturation transitions for α-La (first peak) and β-Lg (second peak) are shown in Figure 5.4. Additional thermograms for vegetable shortening and cocoa butter are shown in Appendix A in Figures A.4 to A.7). Denaturation of these whey proteins in the HPN bars was influenced by bar formulation and storage time, as well as their interactions (Tables 5.13 and 5.14). There were differences in denaturation enthalpy for β-Lg based upon formulation, with initial values of 0.89, 0.62, 0.75 and 0.87 J/g protein for Bars 1, 2, 3 and 4, respectively (Table 5.15). A lower enthalpy value for Bar 2 was not unexpected as MPC consists of only 20% whey proteins. If the denaturation change was only dependent on whey protein content then an even lower denaturation enthalpy would have been reasonable.

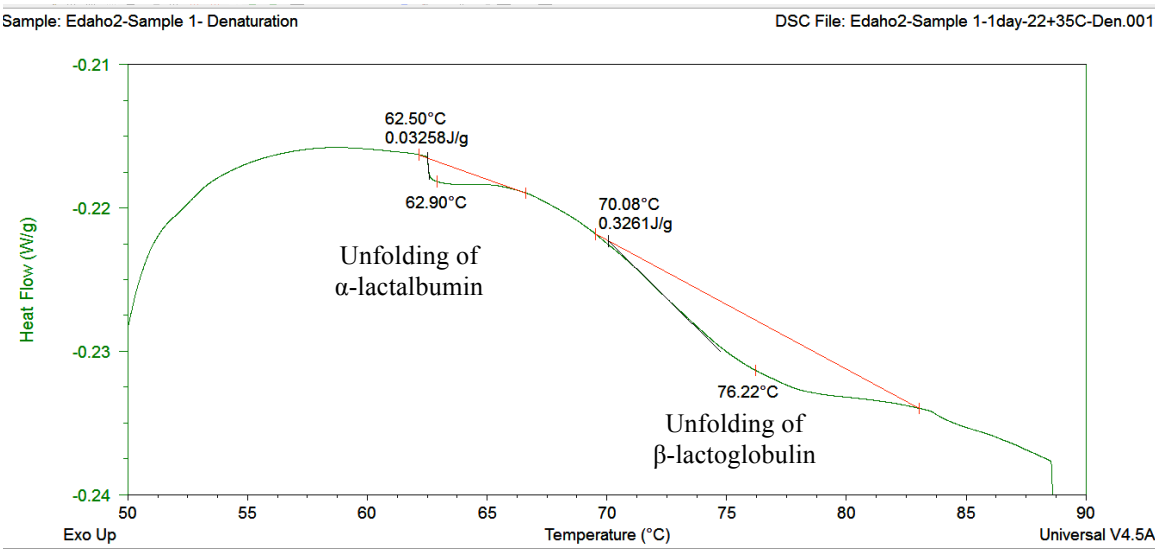

Figure 5.4 Example of a differential scanning calorimeter thermogram of high-protein nutrition bars used for comparing protein denaturation endothermic transitions that were assigned to α-lactalbumin (first peak) and β-lactoglobulin (second peak) (exo up).

Table 5.13 ANOVA of β-lactoglobulin denaturation onset and peak temperatures and enthalpy values of high-protein nutrition bars during storage at 22°C and 35°C.

Type III Tests of Fixed Effects					
Effect	**Numerator DF**	**Denominator DF**	**Onset Temp *P* > F**	**Peak Temp *P* > F**	**Denat Enthalpy *P* > F**
Bar (B)	3	190	<0.0001	<0.0001	<0.0001
Temperature (T)	1	1	0.1131	0.2423	0.0615
B*T	3	190	<0.0001	<0.0001	<0.0001
Storage time (S)	7	190	<0.0001	<0.0001	<0.0001
B*S	21	190	<0.0001	<0.0001	<0.0001
T*S	7	190	<0.0001	<0.0001	<0.0001
B*T*S	21	190	<0.0001	<0.0001	<0.0001

Table 5.14 ANOVA of α-lactalbumin denaturation onset and peak temperatures and enthalpy values of high-protein nutrition bars during storage at 22°C and 35°C.

Type III Tests of Fixed Effects					
Effect	**Numerator DF**	**Denominator DF**	**Onset Temp *P* > F**	**Peak Temp *P* > F**	**Denat Enthalpy *P* > F**
Bar (B)	3	190	<0.0001	0.0002	<0.0001
Temperature (T)	1	1	0.1714	0.1224	0.1363
B*T	3	190	<0.0001	<0.0001	0.1100
Storage time (S)	7	190	<0.0001	<0.0001	<0.0001
B*S	21	190	<0.0001	<0.0001	<0.0001
T*S	7	190	0.2913	<0.0001	<0.0001
B*T*S	21	190	<0.0001	<0.0001	0.0005

Table 5.15 Change in onset and peak temperature and denaturation enthalpy for β-lactoglobulin of a reference[1] high-protein compared to bars in which whey protein isolate was replaced with milk protein concentrate (MPC), sorbitol syrup was replaced with glycerol, or shortening was replaced with cocoa butter during 224 d storage at 22°C.

Time (d)	Bar	Onset Temp. (°C)	Peak Temp. (°C)	ΔH J/g protein
0	1. Reference	80.77^{fghijklmnopq}	82.82^{u}	0.89^{ab}
	2. MPC	78.31^{tuv}	87.48^{jklmn}	0.62^{tu}
	3. Glycerol	76.01^{xy}	80.40^{v}	0.75^{jklmn}
	4. Cocoa Butter	79.01^{qrstuv}	79.97^{v}	0.87^{bc}
6	1. Reference	79.30^{opqrstuv}	85.98^{opqr}	0.90^{a}
	2. MPC	80.42^{hijklmnopqr}	87.93^{hijkl}	0.61^{uv}
	3. Glycerol	75.06^{y}	83.06^{u}	0.73^{mn}
	4. Cocoa Butter	81.50^{cdefghijklm}	85.74^{pqr}	0.86^{cd}
21	1. Reference	80.38^{ijklmnopqr}	85.01^{qrs}	0.82^{ef}
	2. MPC	80.15^{jklmnopqrs}	84.66^{rst}	0.56^{yzA}
	3. Glycerol	81.30^{cdefghijklm}	87.88^{hijklm}	0.68^{qr}
	4. Cocoa Butter	81.03^{efghijklmno}	88.88^{efghij}	0.80^{fg}
42	1. Reference	79.16^{pqrstuv}	84.00^{stu}	0.77^{ghi}
	2. MPC	79.85^{lmnopqrstu}	85.47^{pqrs}	0.64st
	3. Glycerol	79.85^{lmnopqrstu}	88.66^{fghijk}	0.59^{vw}
	4. Cocoa Butter	79.68^{mnopqrstu}	87.69^{hijklm}	0.83^{e}
70	1. Reference	78.41^{stuv}	86.75^{lmnop}	0.75^{klmn}
	2. MPC	79.46^{nopqrstu}	85.53^{pqr}	0.52^{CDE}
	3. Glycerol	79.22^{pqrstuv}	88.03^{hijkl}	0.54^{ABC}
	4. Cocoa Butter	81.60^{cdefghijkl}	86.41^{mnopq}	0.78^{gh}
119	1. Reference	78.12^{uvw}	85.57^{pqr}	0.74^{lmn}
	2. MPC	79.94^{klmnopqrst}	84.99^{qrs}	0.56^{xyz}
	3. Glycerol	81.86^{cdefghij}	89.86^{bcdef}	0.52^{CDE}
	4. Cocoa Butter	82.00^{cdefghi}	88.15^{ghijkl}	0.78^{ghi}
175	1. Reference	80.32^{ijklmnopqr}	87.36^{klmno}	0.78^{ghi}
	2. MPC	80.66^{ghijklmnopq}	86.73^{lmnop}	0.53^{CDE}
	3. Glycerol	82.78^{abcde}	90.56^{abc}	0.55^{yzAB}
	4. Cocoa Butter	80.89^{fghijklmnop}	88.63^{fghijk}	0.74^{lmn}
224	1. Reference	81.94^{cdefghi}	89.82^{bcdef}	0.73^{no}
	2. MPC	81.62^{cdefghijk}	88.95^{efghij}	0.52^{CDE}
	3. Glycerol	82.40^{abcdefg}	91.72^{a}	0.47^{F}
	4. Cocoa Butter	82.15^{bcdefgh}	90.79^{ab}	0.75^{jklmn}

[1]33.9% WPI, 46.7% sorbitol syrup, 19.4% vegetable shortening

$^{abc…xyzABCDEF}$means within columns in Tables 5.15 and A.22 with same letter were not significantly different, α = 0.05

The denaturation enthalpy for Bar 4 was not significantly different ($P > 0.05$) to Bar 1, which was also expected. The only difference the formulation of these bars was that Bar 4 contained cocoa butter rather than shortening, while the protein-water-sorbitol phase was the same.

That Bar 3 had a lower denaturation enthalpy than Bar 1 could be explained by the action of glycerol in stabilizing the native structure of β-Lg, so that there was less unfolding during heating. Having the WPI powder dispersed in glycerol, rather than sorbitol syrup, allowed the β-Lg to unfold at about 2°C lower temperature (Table 5.14). There was a similar lowering on denaturation onset temperature for the α-La denaturation transition (Appendix A, Tables A.21 and A.22).

During storage of the HPN bars, there was a gradual decrease in denaturation enthalpy for both β-Lg and α-La, and a concomitant increase in denaturation temperature (Tables 5.15 and 5.16 and Appendix A, Tables A.21 and A.22). After 224 d storage at 22°C, the β-Lg denaturation enthalpy decreased ~15% for the sorbitol bars and ~40% for the glycerol bars. Denaturation enthalpy had decreased to 0.73, 0.52, 0.47 and 0.75 J/g protein for Bars 1, 2, 3 and 4 respectively. For α-La, denaturation enthalpies decreased about 15% for all the bars to 0.21, 0.20, 0.22 and 0.21 J/g protein, respectively. Similar changes occurred during 90 d storage at 35°C.

Bar Microstructure

Confocal laser scanning images of HPN bars during storage at 22°C and 35°C are shown in Figure 5.5 and Figure 5.6, respectively. In these images, fluorescence from FITC was false colorized as green and occurs when FITC is bound to protein.

Table 5.16 Change in denaturation enthalpy for α-lactalbumin of a reference[1] high-protein compared to bars in which whey protein isolate was replaced with milk protein concentrate (MPC), sorbitol syrup was replaced with glycerol, or shortening was replaced with cocoa butter during 90 d storage at 35°C.

Time (d)	Bar	Onset Temp. (°C)	Peak Temp. (°C)	ΔH J/g protein
0	1. Reference	61.47^{mnopqretuv}	65.07^{vwxy}	0.24^{cdefg}
	2. MPC	62.03^{jklmnopqr}	66.00^{rstuvwx}	0.23^{ijklmnop}
	3. Glycerol	58.51^{zA}	65.76^{rstuvwx}	0.25^{abc}
	4. Cocoa Butter	58.89^{yzA}	64.04^{y}	0.27^{ab}
2	1. Reference	61.25^{nopqrstuvw}	64.90^{xy}	0.23^{ghijklm}
	2. MPC	62.00^{jklmnopqr}	66.48^{opqrstuvw}	0.22^{lmnopqrstu}
	3. Glycerol	61.83^{klmnopqrst}	66.94^{lmnopqrstu}	0.25^{cde}
	4. Cocoa Butter	58.00^{A}	66.38^{pqrstuvwx}	0.25^{cde}
8	1. Reference	62,00^{jklmnopqr}	66.16^{qrstuvwx}	0.23^{fghijk}
	2. MPC	62.24^{jklmnopq}	66.41^{pqrstuvwx}	0.21^{rstuvwx}
	3. Glycerol	63.09^{fghijklmn}	65.77^{rstuvwx}	0.25^{bcd}
	4. Cocoa Butter	59.70^{uvwxyzA}	66.89^{mnopqrstu}	0.23^{fghij}
15	1. Reference	62.00^{jklmnopqr}	67.59^{hijklmnopq}	0.23^{ghijklmn}
	2. MPC	63.52^{efghijkl}	68.95^{defgh}	0.22^{jklmnopq}
	3. Glycerol	63.89^{defghij}	66.00^{rstuvwx}	0.24^{cdef}
	4. Cocoa Butter	60.00^{tuvwxyz}	67.11^{jklmnopqrs}	0.21^{rstuvwxy}
29	1. Reference	61.03^{opqrstuvwx}	66.34^{pqrstuvwx}	0.23^{fghijk}
	2. MPC	62.59^{hijklmnop}	69.27^{defg}	0.21^{pqrstuvw}
	3. Glycerol	63.53^{efghijkl}	68.74^{defghi}	0.24^{defgh}
	4. Cocoa Butter	61.38^{mnopqrstuv}	69.78^{de}	0.20^{vwxyzA}
43	1. Reference	62.94^{ghijklmno}	68.47^{defghijkl}	0.21^{rstuvwxy}
	2. MPC	64.41^{cdefgh}	69.90^{cde}	0.19^{ABC}
	3. Glycerol	61.96^{jklmnopqrs}	68.58^{defghij}	0.22^{klmnopqrst}
	4. Cocoa Butter	61.71^{klmnopqrst}	67.85^{ghijklmnop}	0.22^{mnopqrstuv}
60	1. Reference	65.02^{abcdef}	68.00^{ghijklmno}	0.20^{uvwxyzA}
	2. MPC	63.31^{efghijklm}	70.78^{de}	0.18^{C}
	3. Glycerol	62.90^{hijklmnop}	69.00^{defgh}	0.23^{fghijk}
	4. Cocoa Butter	66.89^{a}	68.00^{ghijklmno}	0.19^{ABC}
90	1. Reference	66.53^{ab}	73.03^{a}	0.19^{zABC}
	2. MPC	63.00^{ghijklmn}	72.08^{ab}	0.17^{D}
	3. Glycerol	62.00^{jklmnopqr}	69.31^{defg}	0.19^{BC}
	4. Cocoa Butter	66.00^{abc}	68.44^{defghijkl}	0.20^{wxyzAB}

[1]33.9% WPI, 46.7% sorbitol syrup, 19.4% vegetable shortening

$^{abc\ldots xyzABC\ldots FG}$ means within columns in Tables 5.16 and A.21 with same letter were not significantly different, α = 0.05

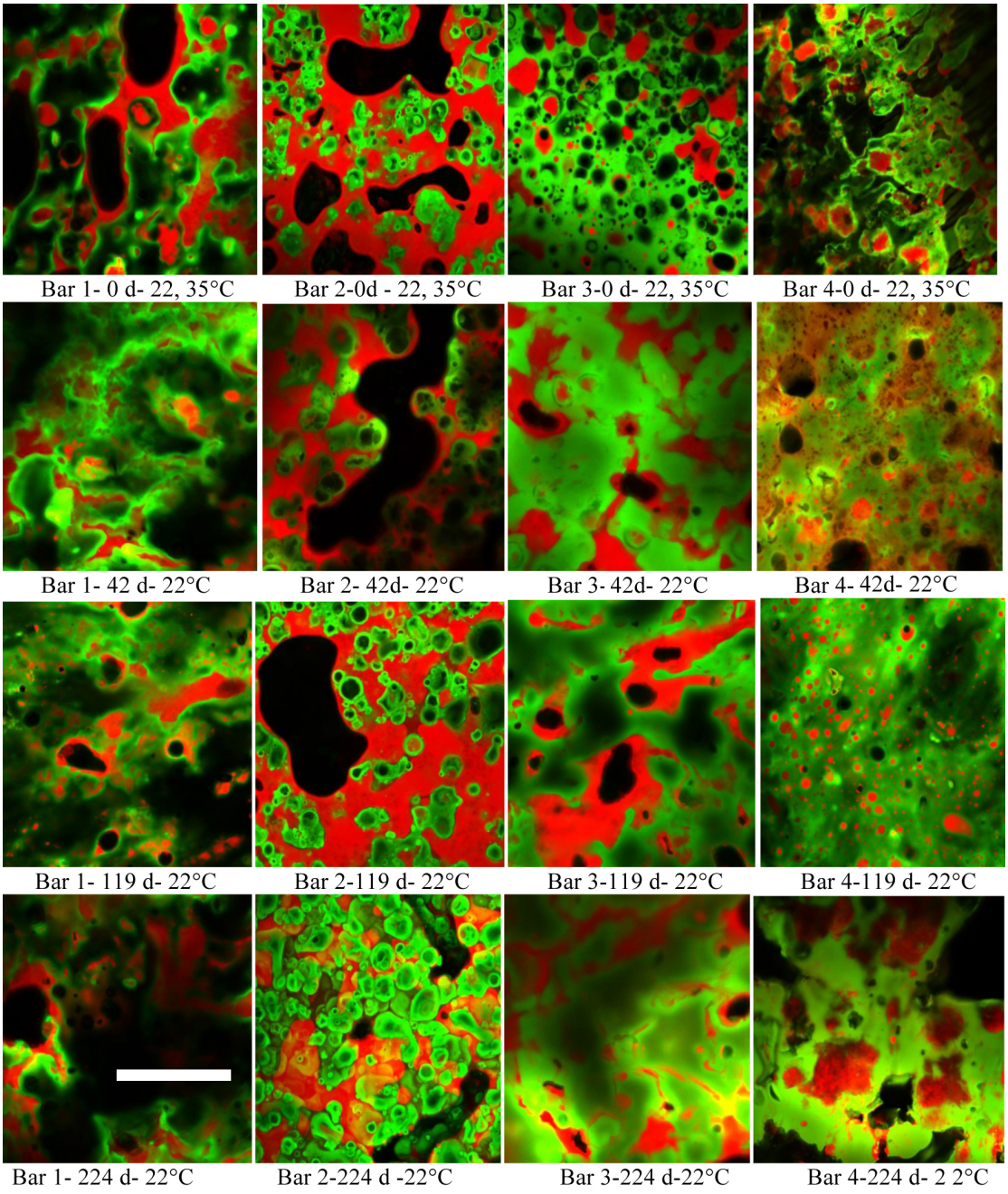

Figure 5.5 Confocal laser scanning micrographs of (1) a reference high-protein nutrition bar (33.9% whey protein concentrate powder, 46.7% sorbitol syrup, 19.4% vegetable shortening) compared to bars in which (2) whey protein isolate was replaced with milk protein concentrate, (3) sorbitol syrup was replaced with glycerol, and (4) shortening was replaced with cocoa butter, during storage for 224 d at 22°C, false colorized with red = lipid, green = protein, black = absence of fluorescence, bar = 200 µm.

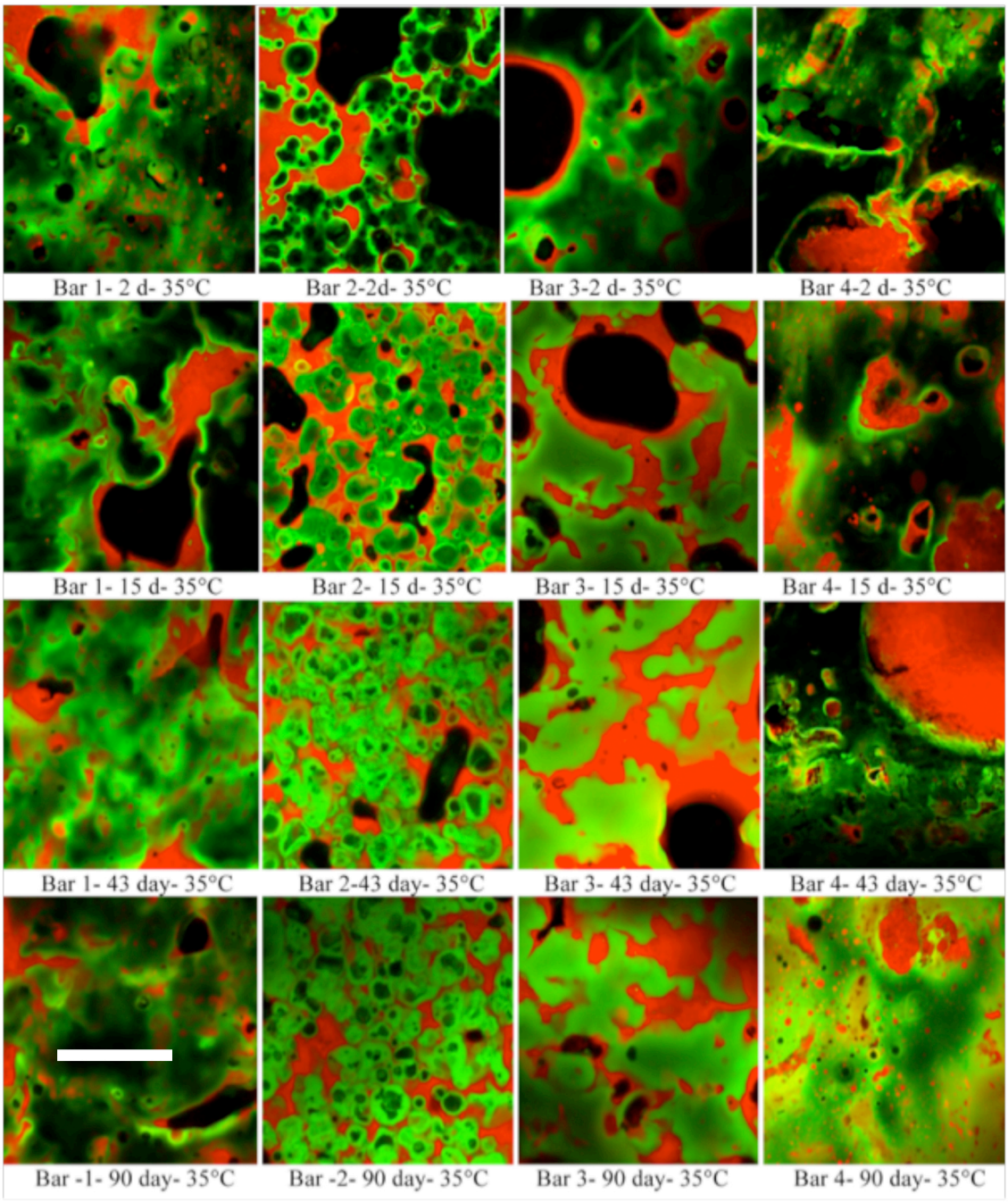

Figure 5.6 Confocal laser scanning micrographs of (1) a reference high-protein nutrition bar (33.9% whey protein concentrate powder, 46.7% sorbitol syrup, 19.4% vegetable shortening) compared to bars in which (2) whey protein isolate was replaced with milk protein concentrate, (3) sorbitol syrup was replaced with glycerol, and (4) shortening was replaced with cocoa butter, during storage for 90 d at 35°C, false colorized with red = lipid, green = protein, black = absence of fluorescence, bar = 200 μm.

Fluorescence from Nile Red was false colorized as red, and occurs when Nile Red is in the presence of lipid material. Black portions of the image occur when there is no fluorescence from that location, from either FITC or Nile Red. The assumption was therefore made that both protein and lipid were absent at those locations.

Upon mixing the bar ingredients together and forming them into bars (d 0), it appeared that most of the protein powder had been dispersed throughout the polyol syrup but was not uniform in protein concentration. There were differences in intensity of FITC fluorescence as shown Figure 5.5, as the green color transitions from bright green (high intensity) though darker areas (lower intensity) into black (absence of fluorescence). This suggests that the level of protein varies within the combined water/polyol/protein phase. It is also important in understanding HPN bar systems, to realize that Bar 1, 2 and 4 contain only about 15% water and that the sorbitol plays a major role as a cosolvent in dispersing the protein powder. In Bar 3, water was virtually absent and glycerol functions as the solvent into which the protein powder was dispersed.

In all images of the bars, there were well-defined locations that were black. These were occasionally spherical, but generally curvilinear in shape, and ranged from very small to very large. Such areas that lack fluorescence from either Nile Red of FITC were determined to be air pockets. In a previous study (McMahon and others 2009) it had been incorrectly assumed that these curvilinear black areas resulted from portions of the polyol/water phase from which proteins had been excluded because of phase separation. In most cases, these black regions are enveloped in red areas representing fluorescence from Nile Red dissolved in the lipid phase of the bars. This concurs with Loveday and

others (2010) and with the tendency for lipids to accumulate at air-water interfaces in foamed materials.

In the bars stored at 22°C, the fluorescence from Nile Red was less evident in Bar 4 compared to the other bars. Bar 4 was made using cocoa butter, which at room temperature (the temperature at which the bars were tempered prior to preparation for CLSM imaging), has a higher solid:fat ratio than does shortening. Consequently, Nile Red solution may not have been as well dispersed and fluorescent. Another interesting observation was that some of the protein powder particles appear to become covered with lipid that prevented their further dispersion. These appeared as small green areas with a red annular ring around them.

After 2 d storage at 35°C (Figure 5.6), it appeared that the protein material in Bars 1 and 4 (both made using WPI and sorbitol syrup) were completely dispersed forming a extensive protein/solvent/cosolvent matrix. The same occurred for Bar 3 within 8 d at 35°C. When stored at 22°C, Bar 1 and 4 were completely dispersed after 6 d, and Bar 3 after 21 d. In contrast, the protein powder in Bar 2 (made using MPC instead of WPI) appeared to remain in particulate form throughout storage, and never formed a continuous protein/solvent/cosolvent phase as in the other bars. This was apparent in the micrographs as circular green regions that had high levels of FITC fluorescence around their perimeter (bright green in appearance) and were dark-green in their center. These would most likely be MPC powder particles that remained separate from each other without any protein network structure between them. Such lack of dissolution of the protein powder to form a continuous network explains the lack of cohesion and crumbly texture of HPN bars in which MPC is the only protein source.

During storage it was observed that the air pockets in the bars were more apparent with longer storage times. It also became more difficult to image regions on the bar sample that were not dominated by large air cavities. Also during storage, the portion of the images that were dark green/black in appearance increased in Bars 1, 3 and 4. There are two explanations for this observation. Either the FITC is not penetrating sufficiently into the protein network areas, or there was a phase separation occurring in which there were portions of the solvent/cosolvent phase from which the proteins were excluded. These large extensive green/black regions were not observed in Bar 2. The CLSM images of the bars were collected from a focal plane that is <20 μm from the sample's surface. This is the surface upon which the FITC-acetone solution was applied and why there would be difference in penetration into the bar over that short distance was not apparent. Even allowing longer times for the fluorophore to penetrate did not eliminate these green/black regions in the CLSM images.

DISCUSSION

Visual Appearance

During manufacture of the four doughs for Bars 1, 2, 3 and 4, differences were observed in the texture and hardness of the bars depending on the ingredients that were used. In general, one minute was needed for mixing the bar ingredients and initially the doughs were soft, malleable, and easily formed into bars. Bar 2 made using MPC as the protein powder, hardened quickly especially on its surface, and tended to lack cohesiveness and were brittle and too crumbly. In comparison, the use of WPI gives a

denser product with strong cohesiveness and adhesiveness as previously shown by Childs and others (2007).

The mixture of WPI, glycerol and shortening (Bar 3) did not form as readily into a dough, and required cooling before it could be put through the bar extruder. At room temperature, the dough for making Bar 3 was too soft and sticky compared with the other doughs. This is explained by the lower viscosity of glycerol compared to sorbitol syrup. In contrast, the dough made with WPI, sorbitol syrup and cocoa butter (Bar 4) was harder than the other bars during manufacture at room temperature, and it took more than one minute to mix the ingredients together in the blender. During storage all bars also underwent changes in both color and consistency. Bar 1 (followed by Bar 4) had the best texture and even though it hardened, it still remained some flexible and was taffy-like in texture. Both of these bars had the least browning during storage.

Air voids. During storage the number and average size of air voids also varied between the bars. This may have been because of contraction of the protein network as a consequence of crosslinking between proteins during storage. Another reason for having an increased number of air voids appear during storage, may be displacement of occluded air from within the protein powder particles as the solvent/cosolvent phase entered the particles, followed by subsequent accumulation of displaced air. The presence of cosolvents in systems can decrease the overall molar volume and adiabatic compressibility of proteins. This occurs because of the ability of the cosolvents to cause expulsion of water-containing voids and increased intramolecular bonding within the protein interior (Almagor and others 1996, 1998), because the cosolvents increase the osmotic stress acting on the proteins (Timasheff, 2002a, 2002b). These changes in the bar

system can occur because of changes in the adiabatic compressibility and volume of the protein molecules themselves, as well as changes in the adiabatic compressibility and density of the solvation layer surrounding the proteins (Taulier and Chalikian 2002).

The presence of more air bubbles in Bar 1 can be related to its continuous protein-solvent-cosolvent phase, while in Bar 2 there were fewer but bigger air voids that formed during storage because of its brittle texture. There were also many non-dissolved (solvated) powder particles observed in Bar 2. In Bar 3 there were numerous small air voids, which were assumed to be because of its low viscosity that allowed for good distribution for glycerol with the WPI powder. Bar 3 thus appeared to have a more extensive continuous proteinaceous phase than the other bars. During storage, Bar 3 reached very high hardness levels. The air voids in Bar 4 were less in number and size (and often appeared circular in form). Compared to Bar 1, Bar 4 had a more homogeneous texture and was harder.

Water

Intermediate-moisture foods generally have water activity in the range 0.6-0.9 (Roos 2001), and the HPN bar systems used in this study were at the bottom of this range. For Bar 3, water activity was well below this range as glycerol was used instead of a syrup that contained 30% water. All of the HPN bars were shelf stable, and no microbial growth would occur during storage at room temperature or 35°C. In general, sorbitol acts as an inhibitor against Maillard browning reactions, and so little browning occurs compared to bars made using reducing sugar syrups (McMahon and others 2009). When glycerol is included in a confectionary-type product, it shifts the maximum rate of

browning to lower water activity. This may also be part of the reason why Bar 3 had lower L* values after storage than Bar 1.

In HPN bars, water can be considered to exist in one of three states: bound water, intermediate water, and free or bulk water (Zhou and others 2008b). Exchange between them can occur at the hydration layer around the protein particle surfaces. Bound water molecules are those that have strong water–ion, and possibly hydrogen–ion and water–dipole interactions, with the protein and sugar/polyol molecules (Kuntz and Kauzmann 1974; Zhou and others 2008b). Intermediate water molecules have weak interactions with the protein surface. They are part of the local domain of water molecules in the region of solution immediately surrounding the protein molecules (McClements 2002).

There was a slight increase in water activity during storage that is consistent with Li and others (2008), McMahon and others (2009) and Banach (2012). This could be expected to lead to bars getting softer over time, but this was not the case. It is important to remember that bound water in bars made using sorbitol syrup accounts for ~99% of the total water. Other researchers have observed that during storage, more water becomes bulk water rather than acting as a plasticizer in the solvent/cosolvent system. This can lead to faster bar hardening and helps explain the increase in water activity (Li and others 2008). In other words, even though the amount of intermediate water is very low, its presence around the surface of proteins is key to the properties of the HPN bars. As shown with the increase in water activity during storage, an increase in bulk water can be considered a loss of ability of solvent molecules to maintain protein flexibility.

Ruan and Chen (1998) reported that bar hardness is related to change in the water molecules' mobility caused by a migration of plasticizer water from the polyol syrup to

the protein during storage. This causes formation of molecular linkages as hypothesized by (Gallo-Torres 2003). Such migration would cause the amount of bound water in the product to increase thereby reducing the amount of intermediate water available to act as a plasticizer (Li and others 2008). This was observed for Bars 1, 2 and 4 made using sorbitol syrup, with bound water increasing by ~0.02 g/100 g solids during storage. Having a lack of water molecules associated with the protein surface may also allow increased interaction between neighboring amino acids to occur. This could allow the formation of disulfide bonds that has been suggested as a mechanism of bar hardening (Zhou and others 2008a, 2008b).

Other researchers (Lin and others 2006) reported no change in water activity of HPN bars as they hardened, and have suggested that the amount of water available to act as a plasticizer could affect the glass transition process. However, no glass transitions were observed in the DSC thermograms of the bars included in this study.

What appears to be happening in the bars during storage, is that initially water (and sorbitol) move into the dry protein powder particles as part of the dough making process and solvate the proteins. Following this initial solvation, and as the solvation process continues during the first few days, there is a slight decrease in bulk water (see Appendix A, Table A.6 and A.7). This probably comes about as water contained within the protein powders begins to interact with sorbitol molecules. Then during storage, as proteins aggregate (see Figure 5.7) under the influence of solvent/cosolvent interactions, the amount of bulk water increases. These may be water molecules that are occluded as a group within a pocket of the protein, that are consequently shielded from the sorbitol molecules. Some of these water molecules may be interacting with the protein

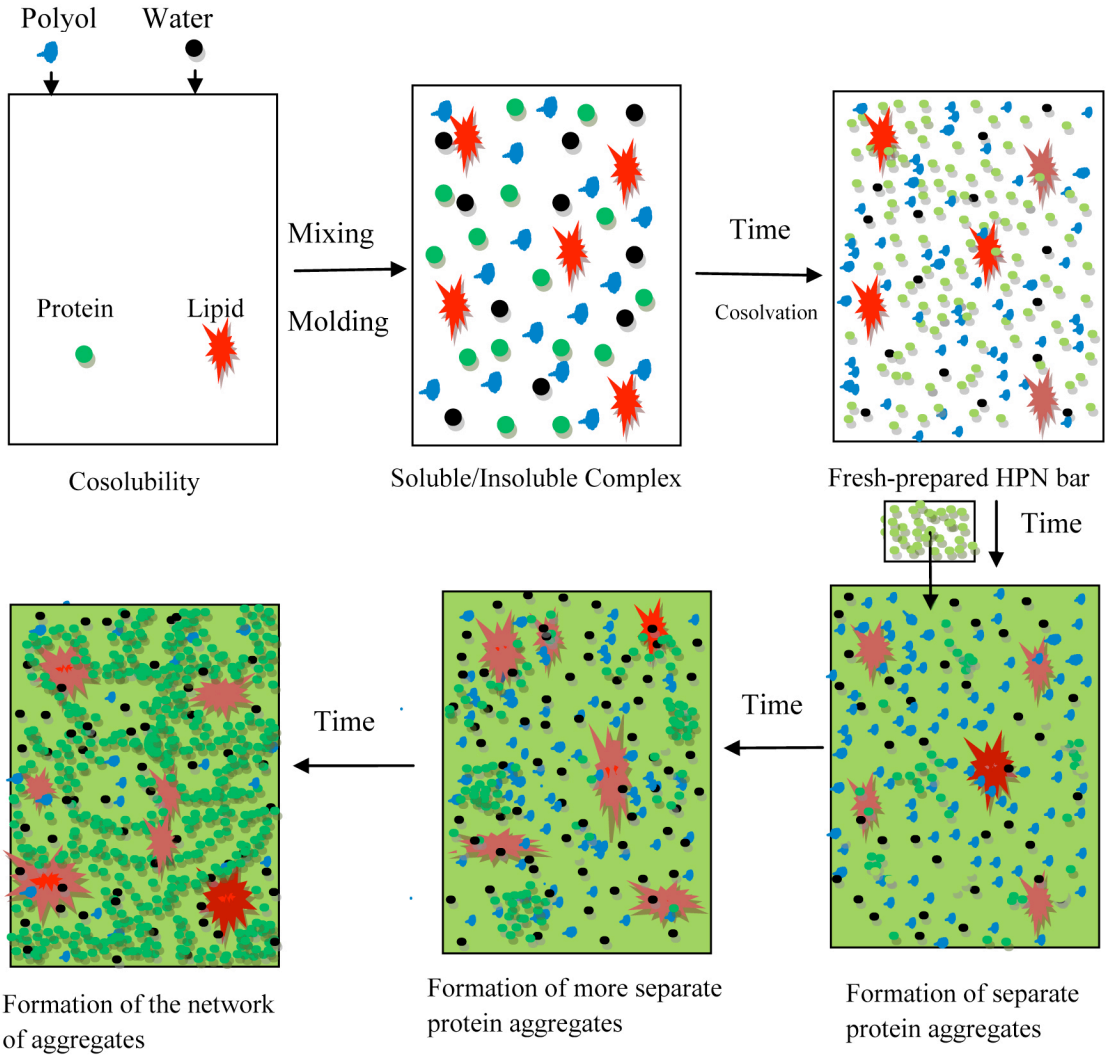

Figure 5.7 Schematic representation of formation of protein aggregates in high-protein nutrition (HPN) bars as a cause of bar hardening.

and would be classified as bound water. Other water molecules may only be interacting with water molecules and so would be classified as bulk water.

Browning

One consideration for hardening of HPN bars has been that it may be associated with Maillard browning. This could occur in a similar manner that Maillard reactions in WPI gels have been associated with strengthening of the gel (Rich and Foegeding 2000). In theory, inhibition of Maillard browning would slow HPN bar hardening. Maillard browning products can increase the rate of formation of disulfide bonds. They can also increase hydrophobic network formation between proteins that then expels water that had previously been associated with local protein surface domains (Anema and others 2006; Le and Deeth 2011). However, when Maillard browning was inhibited in HPN bars by sulfite addition, hardening was not affected (Baier and others 2007). Furthermore, McMahon and others (2009) in a study on hydrolyzed WPI in HPN bars, found that there was no relationship between browning reaction and bar hardening. Bars made using hydrolyzed WPI bars had more brown color but were softer than bars made using WPI.

Solvent/Cosolvent

McMahon and others (2009) had proposed that a phase separation into protein-rich and cosolvent-rich (protein-free) phases was the cause of hardening of HPN bars. Using hydrolyzed WPI was an effective way to inhibit bar hardening as this retarded phase separation. This conclusion was based on CLSM images of bars during storage, and those areas they considered to be indicative of phase separation have now been shown in this study to be air voids. The correlation may be that large air voids become

more prevalent as the bars become harder. Using hydrolyzed WPI helps keep the bars soft, and so fewer air bubbles are formed and they remain small in size. With a newer confocal microscope it was easier to demonstrate that air bubbles caused the black regions in the images, rather than pockets of phase separated polyol (data not shown).

When sorbitol syrup is used as the carbohydrate source in HPN bars, it contributes both water and sorbitol molecules as the solvent/cosolvent mixture. This syrup contains 30% water and 70% sorbitol. There is also a small amount of water contained in the protein powders. In the sorbitol syrup there is no intermediate water and no bulk water (see Figure A.3). Any water that is present is strongly interacting via hydrogen bonds with the surrounding sorbitol molecules. After making up the doughs for the HPN bars, ~99.0% of the water still exists as bound water. There is some intermediate water (~0.4%) and some bulk water (~0.1%), and these are most likely associated with the proteins and would be shielded from interacting with sorbitol molecules.

When a solvent/cosolvent mixture such as sorbitol syrup is mixed with a protein, there is a preferential exclusion of the sorbitol molecules from the immediate vicinity of the protein surfaces because of the larger size of sorbitol compared to water. It would be within this exclusion zone that the intermediate water molecules are interacting with protein moieties. Using glycerol as the carbohydrate component produces a very different environment around the protein surfaces and causes very rapid HPN bar hardening. There is essentially no exclusion layer that preferentially contains water molecules surrounding the protein surfaces, and so glycerol interacts directly with the protein moieties. Such lack of water molecules associated with the protein surface could allow neighboring amino acids to form disulfide bonds leading to protein aggregation as

suggested by Zhou and others (2008a, 2008b). It could also allow increased entropic effects causing coalescence of hydrophobic domains, that then lead to protein aggregation and bar hardening. Systems containing glycerol are more susceptible to entropic effects because the number of possible interactions between glycerol molecules is less than that which can occur between water molecules. This suggests that the rapid hardening of Bar 3 (made using glycerol, WPI and shortening) is a consequence of extensive hydrophobic-induced aggregation of the proteins.

SUMMARY

Phase Separation. From these experiments, it was demonstrated that McMahon and others (2009) were incorrect in assigning the black regions observed in CLSM images of HPN bars to being sorbitol syrup that was devoid of protein. These black regions have no fluorescence from FITC or Nile Red because they are air pockets that become more evident during storage, especially as the bars harden. Therefore, there is no evidence to support a phase separation occurring between the sorbitol and the proteins, and this can be eliminated as a mechanism responsible for bar hardening.

Using MPC as the Protein Powder instead of WPI. When MPC is used as the protein powder instead of WPI, the bars start out slightly softer than bars made using WPI, and harden at a slightly faster rate. Although, even after 60 d storage at 35°C or 175 d at 22°C the bars have similar hardness scores. The major difference is that the MPC bars were more brittle and fractured during the penetration test while the WPI bars were more pliable. The MPC powder remains in particulate form in comparison to the WPI bars in which the protein appears to be evenly spread throughout the sorbitol syrup.

Having the MPC remain in particles that are10 to 50 μm in size imparts a crumbly texture to the bars.

Using Glycerol instead of Sorbitol Syrup. The effect on hardness of using glycerol instead of sorbitol syrup in the bars was surprising as glycerol has a much lower viscosity, and is often used as a plasticizing agent. The bars made using glycerol were initially much softer. However, they underwent a rapid hardening after the initial few days of storage during which the dry protein powder becomes solvated after mixing the components together to form a dough. This suggests that there are interactions taking place between glycerol and the surface of the protein molecules that are promoting aggregation of the proteins.

Using Cocoa Butter instead of Shortening. Using cocoa butter increased the initial hardness of the bars. This was assumed to be because of its higher solid to liquid ratio compared to shortening. Having a more solid lipid component did not appear to influence hardening during storage. The bars made with cocoa butter followed the same trend in hardening as the bars made using shortening.

Browning. The extent of browning during storage of the bars was minimal, with the bars still only having a slight tan-cream color after storage. There was no practical difference in the color of the bars made using glycerol compared to the reference bars made using sorbitol. Yet, the bars made using glycerol were four times harder than the reference bars after storage. Having no relationship between hardness and extent of browning has been previously observed (McMahon and others 2009).

Water Activity. A difference between the bars made using glycerol and other bars was that the initial water activity was lowered to 0.12 compared to ~0.69. Water

activity increased slightly during storage for all bars. This corresponded to a small decrease in intermediate water with a concomitant small increase in both bound water and bulk water. In the bars, ~99.0% of the water is bound (i.e., unfreezable at -40°C) and these changes suggest some movement of water related to the protein surfaces.

Protein Aggregation. Hardening of HPN bars occurs when an extensive protein network structure develops. Why the bars with glycerol hardened more than bars made with sorbitol syrup is not understood. Presumably it is related to how glycerol interacts with the protein molecules at their surfaces that subsequently promotes aggregation.

Recommendations for Future Research. For Phase 2 of this research, further modifications of the HPN bar formulation will be undertaken to provide insight into how protein aggregation can be promoted or retarded. This should include:

- whether the lower protein and higher lactose level of MPC compared to WPI influences hardening,
- mixtures of proteins to determine what level of MPC can be added without having problems with a crumbly brittle texture,
- mixtures of sorbitol syrup and glycerol to determine if hardening increases with amount of glycerol added.
- whether calcium is involved in hardening,
- does the lipid portion play any role in hardening, or does it just function as a semi-solid filler, and
- whether adding a surfactant influences hardening.

CHAPTER 6.

PHASE 2: AN EXPLORATION OF HOW COMBINING DIFFERENT PROTEINS, POLYOLS AND LIPIDS AND OTHER ADDITIVES IN HPN BAR FORMULATIONS INFLUENCES HARDENING

INTRODUCTION

After the set of HPN bars made during Phase 1, were stored and analyzed, and problems observed in hardening (especially when using glycerol in place of sorbitol syrup) and crumbliness (when using MPC), a series of tests were performed to investigate the effect of changing individual components of the bars. This was done using 33-g batches of each formulation that were made in duplicate. Initial bar hardness, and hardness after storage at 35°C were measured as well as color and water activity. The aim of these experiments was to see what changes in formulation had large impacts on retarding bar hardening. From the results of these experiments, a series of formulations could then be selected for a more in-depth study (i.e., for Phase 3 experiments).

The formulation changes that were explored included:

- adding lactose to account for the higher lactose and lower protein content of MPC compared to WPI,
- varying the amount of protein powder added, to determine the maximum limits on protein content and how this was influenced by bar formulation, based on whether WPI or MPC was used,
- testing to see whether adding a surfactant or chelating calcium, modified the proteins in such a way that they would retard protein aggregation and bar hardening,

- mixing shortening and cocoa butter in various ratios, as well as changing to a liquid oil rather than a semi-solid fat, and whether there are any favorable or unfavorable interactions between lipid and protein or polyol that influence bar hardening,
- making bars in with glycerol and sorbitol syrup mixed in different ratios, and seeing if hardening is influenced by using WPI or MPC, either alone or combined,
- seeing if the protein aggregation that brings about bar hardening is related to disulfide bond formation by adding a reducing agent to inhibit disulfide bond formation.

RESULTS AND DISCUSSION

Lactose (MPC Compared to WPI)

When substituting MPC for WPI in bar formulations, as well as replacing some of the whey proteins with casein (80% of protein in MPC is casein), there is also a slight decrease in protein level and an increase in lactose. The WPI contains 90% protein and about 1% lactose, while MPC contains 80% protein and about 6% lactose.

Increasing the amount of WPI powder in bar formulations from 33.9% to 37.1% increased initial bar hardness by 50%, from 41 to 62 g-force as expected (see #1 and #4 in Table 6.1). Extent of hardening during 29 d at 35°C increased even more and the higher protein bars were about twice as hard. The extent of color change also increased as more amino groups would be available to participate in Maillard browning reactions.

Adding lactose into bar formulations increased hardness and relative change in hardness (%RC) when it replaced the solvent/cosolvent (see #2 and #3 in Table 6.1), but didn't impact color change. When lactose replaced the protein powder (#7 in Table 6.1), the bars were initially softer (23 g-force), and remained softer during storage with

Table 6.1 Effect of lactose and protein levels on mean (SD in bold) hardness, relative change in hardness (%RC[1]) and color change (ΔE[2]) of high-protein nutrition bar model systems made using whey protein isolate (WPI), sorbitol syrup (Sorb) and shortening (Short) during storage at 35°C (n=2).

Test #	Bar Composition	D 1	Day 29		
		Hardness (g-force)	Hardness (g-force)	%RC	ΔE
1	33.9% WPI 46.7% Sorb 19.4% Short (Standard)	41.2 **1.6**	328.8 **7.9**	698.3 **11.0**	16.7 **1.0**
2	33.6% WPI 46.3% Sorb 1.0% Lactose 19.1 Short	49.1 **2.7**	575.9 **9.3**	1074.2 **45.2**	17.9 **1.3**
3	32.3% WPI 44.5% Sorb 4.8% Lactose 18.4% Short	52.6 **3.1**	473.4 **7.5**	801.1 **39.1**	21.9 **1.0**
4	37.1% WPI 44.5% Sorb 18.4% Short	61.7 **2.3**	815.5 **11.3**	1223.0 **66.8**	21.4 **0.8**
5	36.7% WPI 44.1% Sorb 1% Lactose 18.2% Short	56.7 **2.7**	791.2 **12.9**	1296.5 **43.5**	20.4 **1.0**
6	35.4% WPI 42.5% Sorb 4.6% Lactose 17.5% Short	94.9 **3.4**	654.6 **14.7**	590.0 **9.1**	21.8 **1.3**
7	28.9% WPI 46.8% Sorb 5% Lactose 19.3% Short	22.7 **1.0**	261.4 **8.1**	1051.9 **14.7**	19.9 **0.8**

[1]%RC = [(Hardness – Initial Hardness) / Initial Hardness] x 100.

[2]$\Delta E = [(a^* - a^*_0)^2 + (b^* - b^*_0)^2 + (L^* - L^*_0)^2]^{1/2}$

hardness of 261 g-force after 29 d at 35°C. However, when considered on the basis of relative change there was more hardening. For all the samples with added lactose, there was a trend for a greater color change when 5% lactose was added to replace WPI.

From this it was concluded that the additional lactose added when using MPC instead of WPI, may make the bars slightly softer at the time of manufacture but does not slow down the rate of hardening. Increased browning during storage appears more dependent on the level of amino groups available to participate in browning reactions, than on addition of lactose.

Protein Level

Increasing the amount of protein included in a HPN bar increased the initial bar hardness, and the rate of hardening also increased (Table 6.2). There was also an increase in browning reactions with more color change occurring. The same changes occur whether the protein powder being used was WPI or MPC.

Addition of Surfactant

Adding the polysorban surfactant Tween 20 into the bar formulation increased the initial bar hardness for Bars 1, 2 and 4, all of which were made using sorbitol syrup. In contrast, it decreased the hardness of Bar 3 made using glycerol (Table 6.3). Addition of Tween 20 had no major effect on hardening rate. The bars with Tween 20 were comparable to those with the same formulation but without the added surfactant, after 43 d storage at 35°C.

Table 6.2 Effect of protein levels on mean (SD in bold) hardness, relative change in hardness (%RC[1]) and color change (ΔE^2) of high-protein nutrition bar model systems made using whey protein isolate (WPI) or milk protein concentrate (MPC), sorbitol syrup (Sorb) and shortening (Short) during storage at 35°C (n=2).

Test #	Bar Composition	D 1	Day 29		
		Hardness (g-force)	Hardness (g-force)	%RC	ΔE
1	33.9% WPI 46.7% Sorb 19.4% Short (Standard)	41.2 **1.6**	328.8 **7.9**	698.3 **11.0**	16.7 **1.0**
2	37.1% WPI 44.5% Sorb 18.4% Short	61.7 **2.3**	815.5 **11.3**	1223.0 **66.8**	21.4 **0.8**
3	31.4%WPI, 48.5% Sorb 20.1% Short	32.7 **0.8**	199.0 **5.5**	509.0 **32.7**	14.5 **0.8**
4	33.9% MPC 46.7% Sorb 19.4% Short (Standard)	65.8 **3.4**	530.2 **8.5**	707.2 **54.5**	9.4 **0.6**
5	37.1% MPC 44.5% Sorb 18.4% Short	135.6 **3.8**	1067.3 **12.3**	687.3 **13.1**	13.7 **0.7**
6	31.4% MPC 48.5% Sorb 20.1% Short	38.1 **2.1**	333.2 **9.1**	775.3 **25.0**	11.6 **0.8**

[1]%RC = [(Hardness – Initial Hardness) / Initial Hardness] x 100

[2]$\Delta E = [(a^* - a^*_0)^2 + (b^* - b^*_0)^2 + (L^* - L^*_0)^2]^{1/2}$

Table 6.3 Effect of adding Tween-20 on the mean (SD in bold) water activity (A_w), hardness, relative change in hardness (%RC[1]) and color change (ΔE^2) of high-protein nutrition bar model systems made using whey protein isolate (WPI) or milk protein concentrate (MPC) with sorbitol syrup (Sorb) or glycerol (Glyc) and vegetable shortening (Short) or cocoa butter (CB) based on the formulations used in Chapter 5 and described in Table 3.2, during storage at 35°C (n=2).

Test #	Bar Composition	Day 1		Day 43			
		A_w	Hardness (g-force)	A_w	Hardness (g-force)	%RC	ΔE
1	33.9% WPI 46.7% Sorb 19.4% Short	0.641 **0.001**	43.7 **1.1**	0.673 **0.001**	396.2 **6.4**	807.1 **38.0**	21.0 **1.3**
2	33.9% MPC 46.7% Sorb 19.4 Short	0.668 **0.001**	69.6 **3.1**	0.690 **0.001**	529.5 **7.4**	661.3 **23.5**	12.1 **0.6**
3	33.9% WPI 46.7% Glyc 19.4% Short	0.116 **0.004**	18.2 **0.4**	0.128 **0.003**	1194.7 **18.5**	6465 **51.3**	24.6 **1.3**
4	33.9% WPI 46.7% Sorb 19.4% CB	0.632 **0.006**	110.5 **5.9**	0.668 **0.001**	599.1 **10.3**	443.3 **38.5**	28.2 **1.4**
5	33.9% WPI 45.7% Sorb 19.4% Short 1% Tween20	0.636 **0.006**	58.3 **2.8**	0.671 **0.004**	370.5 **8.2**	537.1 **44.3**	27.0 **0.6**
6	33.9% MPC 45.7% Sorb 19.4% Short 1% Tween20	0.652 **0.004**	89.9 **3.0**	0.682 **0.001**	485.7 **11.0**	440.3 **5.6**	21.8 **1.1**
7	33.9% WPI 45.7% Glyc 19.4% Short 1% Tween20	0.076 **0.009**	15.6 **0.1**	0.126 **0.004**	1052.5 **13.6**	6669 **118.1**	34.0 **0.8**
8	33.9% WPI 45.7% Sorb 19.4% CB, 1% Tween20	0.617 **0.007**	133.9 **8.2**	0.666 **0.002**	569.5 **5.2**	326.0 **22.2**	45.0 **1.1**

[1]%RC = [(Hardness – Initial Hardness) / Initial Hardness] x 100

[2]$\Delta E = [(a^* - a^*_0)^2 + (b^* - b^*_0)^2 + (L^* - L^*_0)^2]^{1/2}$

Table 6.4 Effect of adding disodium citrate on the mean (SD in bold) water activity (A_w), hardness, relative change in hardness (%RC[1]) and color change (ΔE^2) of high-protein nutrition bar model systems made using milk protein concentrate (MPC) with sorbitol syrup (Sorb) and vegetable shortening (Short) based on Bar 2 formulation used in Chapter 5 and described in Table 3.2, during storage at 35°C (n=2).

Test #	Bar Composition	Day 1		Day 43			
		A_w	Hardness (g-force)	A_w	Hardness (g-force)	%RC	ΔE
1	33.9% MPC 46.7% Sorb 19.4% Short	0.659 **0.003**	71.3 **2.6**	0.682 **0.004**	581.8 **7.6**	717.3 **40.7**	14.4 **0.6**
2	33.2% MPC 46.1% Sorb 2.0% Water, 18.7% Short	0.698 **0.003**	209.3 **4.0**	0.712 **0.003**	537.7 **7.4**	157.0 **8.3**	14.8 **0.4**
3	32.9% MPC 45.7% Sorb 1.0% Citrate in 2.0% Water 18.4% Short	0.685 **0.001**	139.0 **4.0**	0.707 **0.003**	501.0 **10.0**	260.7 **17.5**	17.0 **1.0**

[1]%RC = [(Hardness – Initial Hardness) / Initial Hardness] x 100

[2]$\Delta E = [(a^* - a^*_0)^2 + (b^* - b^*_0)^2 + (L^* - L^*_0)^2]^{½}$

Calcium Chelation using Citrate.

Adding 2% (wt/wt) of a 1% (wt/vol) sodium citrate solution to chelate some of the calcium present in MPC, increased the initial hardness (#3) compared to a control bar (Test #1) (Table 6.4). During 43 d storage at 35°C, the rate of hardening was lower but the bars reached almost the same hardness (501 g-force compared to 582 g-force) as Test #1 (i.e., Bar 2 formulation described in Chapter 5). An even greater increase in initial bar hardness occurred when water was added (Test #2) instead of the citrate solution. Bar hardening does not therefore appear to involve calcium-mediated reactions.

Type of Oil

Shortening and Cocoa Butter. Making bars in which vegetable shortening was replaced 20%, 50%, 80% or 100% with cocoa butter, increased the initial hardness of the bars from 32.9 g-force to 104.6 g-force, depending on the amount of cocoa butter used (Table 6.5). Similar amounts of hardening occurred during storage. The bars containing cocoa butter, with its higher solid:liquid fat ratio at room temperature, remaining slightly harder. After 43 d at 35°C, bar hardness ranged from 405 g-force for the bar with shortening to 530 g-force for the bar made using only cocoa butter.

Soybean Oil. Bar formulations based on Bar 1 and Bar 3 from Chapter 5 were compared with bars in which the vegetable shortening was replaced with soybean oil (newly purchased) or soybean oil that was highly oxidized (~2 years old). All bars used WPI as the protein source while Bar 1 used sorbitol syrup and Bar 3 used glycerol.

Replacing shortening with soybean oil decreased initial hardness (see Test #2 versus #1 and #5 versus #4 in Table 6.6), which was expected as a solid fat was being replaced by a liquid oil. In the sorbitol bar (Test #2), the same hardness was reached after 43 d storage at 35°C whether shortening or soybean oil was used. This indicates that the hardening during storage is a function of a protein network being formed, and that the lipid plays little, if any, role in this network other than as a filler and whether it is present as a solid or a liquid makes no difference.

In contrast, when glycerol was the carbohydrate source in the bars, the bar containing soybean oil was harder after 43 d than the bar containing shortening (Table 6.6). The reason for this is unclear, and perhaps having a liquid oil filler increases the interactions between proteins and glycerol that leads to bar hardening. The effect of

Table 6.5 Effect of substituting cocoa butter (CB) for shortening on the mean (SD in bold) water activity (A_w), hardness, relative change in hardness (%RC[1]) and color change (ΔE^2) of high-protein nutrition bar model systems made using whey protein isolate (WPI) with sorbitol syrup (Sorb) or glycerol (Glyc) and vegetable shortening (Short) based on Bar 1 and 4 formulation used in Chapter 5 and described in Table 3.2, during storage at 35°C (n=3).

Test #	Bar Composition	Day 1		Day 43			
		A_w	Hardness (g-force)	A_w	Hardness (g-force)	%RC	ΔE
1	33.9% WPI 46.7% Sorb 19.4 % Short	0.633 **0.174**	32.9 **1.5**	0.671 **0.003**	405.4 **3.4**	1134.1 **62.9**	20.8 **0.7**
2	33.9% WPI 46.7% Sorb 15.5 % Short 3.9 % CB	0.619 **0.003**	35.8 **1.5**	0.668 **0.668**	420.9 **5.3**	1076.7 **34.9**	19.1 **0.4**
3	33.9% WPI 46.7% Sorb 9.7 % Short 9.7 % CB	0.624 **0.002**	86.3 **1.7**	0.671 **0.002**	447.6 **4.3**	418.7 **5.3**	21.7 **0.6**
4	33.9% WPI 46.7 % Sorb 3.9 % Short 15.5% CB	0.623 **0.002**	96.5 **2.8**	0.670 **0.002**	465.3 **4.6**	382.4 **11.9**	25.7 **0.8**
5	33.9% WPI 46.7% Sorb 19.4% CB	0.624 **0.003**	104.6 **3.7**	0.667 **0.002**	530.5 **5.8**	407.7 **13.1**	23.6 **0.1**

[1]%RC = [(Hardness – Initial Hardness) / Initial Hardness] x 100

[2]$\Delta E = [(a^* - a^*_0)^2 + (b^* - b^*_0)^2 + (L^* - L^*_0)^2]^{1/2}$

Table 6.6 Effect of substituting soybean oil (including an oxidized oil) for shortening on the mean (SD in bold) water activity (A_w), hardness, relative change in hardness (%RC[1]) and color change (ΔE^2) of high-protein nutrition bar model systems made using whey protein isolate (WPI) with sorbitol syrup (Sorb) or glycerol (Glyc) and vegetable shortening (Short) based on Bar 1 and 3 formulation used in Chapter 5 and described in Table 3.2, during storage at 35°C (n=2).

Test #	Bar Composition	Day 1		Day 43			
		A_w	Hardness (g-force)	A_w	Hardness (g-force)	%RC	ΔE
1	33.9% WPI 46.7% Sorb 19.4% Short	0.638 **0.004**	39.2 **0.8**	0.670 **0.002**	397.0 **4.4**	913.1 **33.1**	21.3 **1.1**
2	33.9% WPI 46.7% Sorb 19.4% Raw Soybean Oil	0.641 **0.002**	36.4 **1.2**	0.632 **0.001**	391.1 **3.0**	976.7 **43.8**	26.2 **0.8**
3	33.9% WPI 46.7% Sorb 19.4% Oxid Soybean Oil[3]	0.628 **0.001**	54.7 **1.4**	0.665 **0.001**	492.5 **3.7**	800.6 **16.5**	45.9 **1.0**
4	33.9% WPI 46.7% Glyc 19.4% Short	0.104 **0.005**	18.3 **0.4**	0.126 **0.004**	1198.4 **11.5**	6468 **190.4**	25.7 **1.1**
5	33.9% WPI 46.7% Glyc 19.4% Raw Soybean Oil	0.074 **0.006**	7.4 **0.1**	0.142 **0.003**	1407.4 **14.8**	18924 **564.3**	28.5 **1.0**
6	33.9% WPI 46.7% Glyc 19.4% Oxid Soybean Oil	0.074 **0.005**	9.3 **0.3**	0.127 **0.003**	1809.0 **16.2**	19363 **766.0**	49.3 **1.0**

[1]%RC = [(Hardness – Initial Hardness) / Initial Hardness] x 100.

[2]$\Delta E = [(a^* - a^*_0)^2 + (b^* - b^*_0)^2 + (L^* - L^*_0)^2]^{1/2}$

using oxidized oil was interesting as it accelerated the rate of bar hardening in conjunction with glycerol. After 43 d storage, Test #4, #5 and #6 had hardness values of 1198, 1407 and 1809 g-force, respectively. Less effect of oxidized oil was observed in conjunction with sorbitol syrup. Final hardness for Test #1, #2 and #3 was 397, 391 and 492 g-force, respectively.

Oxidation of oils generates compounds that have hydrophilic components that may allow interactions with proteins that may further lead to hardening. Oxidation of lipids does not necessarily imply breakage of the lipid chains, and formation of covalent bonds between long chain oxidized lipids and amino acids and proteins can occur (Gardner and others 1976; Hidalgo and Zamora 1995; Francisco and others 2000). The bars containing oxidized oils also had greater color change during storage (Table 6.5). This comes about because of the production of reactive carbonyl groups during oxidation that can then reactive with amino groups as part of the Maillard browning, or through other hydrophilic or hydrophobic interactions. It is difficult to separate all of an oxidized lipid mixed with a protein using methods that disrupt hydrogen bonds (Francisco and others 2000). The remaining lipid can only be removed from the protein using chemical treatment, and such oxidized oil is likely to be bound to the protein by covalent bonds

Also in Chapter 5, it was observed that there was more browning of the bars made using cocoa butter (Bar 4) than the reference bar (Bar 1) made with shortening. In comparison to shortening, cocoa butter has only half the amount of unsaturated fatty acids. Therefore, on that basis cocoa butter would be expected to be less reactive. However, shortening has antioxidants specifically added to it to prevent oxidation during

its storage, and these may be having a secondary effect of minimizing reactions between the lipid and protein.

Sorbitol and Glycerol

WPI and Glycerol. Substituting 20% or 30% glycerol for sorbitol syrup in Bar 1 formulation from Chapter 5, decreased the initial bar hardness from 43.5 to 39.9 and 35.4 g-force, respectively (Table 6.7). When a 50:50 mixture of sorbitol syrup and glycerol was used the bar hardness increased to 47.1 g-force. At higher glycerol levels (i.e., 80% substitution), bar hardness was again lower and was similar to bars made with only glycerol instead of sorbitol syrup. The increased hardness with the 50:50 mixture (Test #4 in Table 6.7) is an indication that solvent/cosolvent ratios influence their interactions with proteins. At low concentration, glycerol as a low level cosolvent can act as a plasticizer in relation to the high viscosity sorbitol syrup, and thus lower bar hardness. When using 80% glycerol (Test #5), the glycerol is at a high enough level that it functions as the solvent with sorbitol and water as cosolvents. Then the low viscosity of the glycerol becomes the predominant determinant of bar hardness.

Substitution of 20% and 30% of the sorbitol syrup with glycerol in WPI bars slowed down the rate of bar hardening. After 43 d storage at 35°C, bar hardness was 266.8 and 196.5 g-force, respectively, compared to 388.1 g-force for the control bar (Table 6.7). The change in hardness was thus lower with a %RC of 570% and 455%, compared to 793%. When the glycerol proportion was increased to 50%, the action of glycerol as the predominant solvent had the greater effect and the bars with 50%, 80% or 100% glycerol had %RC values of 718%, 3625% and 6399%, respectively.

Table 6.7 Effect of substituting glycerol for sorbitol syrup on the mean (SD in bold) water activity (A_w), hardness, relative change in hardness (%RC[1]) and color change (ΔE^2) of high-protein nutrition bar model systems made using whey protein isolate (WPI) with sorbitol syrup (Sorb) or glycerol (Glyc) and vegetable shortening (Short) based on Bar 1 and 3 formulation used in Chapter 5 and described in Table 3.2, during storage at 35°C (n=3).

Test #	Bar Composition	Day 1		Day 43			
		A_w	Hardness (g-force)	A_w	Hardness (g-force)	%RC	ΔE
1	33.9% WPI 46.7% Sorb 19.4% Short	0.644 **0.002**[7]	43.5 **0.8**	0.676 **0.002**	388.1 **4.3**	793.2 **23.3**	22.5 **0.1**
2	33.9% WPI 37.4% Sorb 9.3% Glyc 19.4% Short	0.536 **0.002**	39.9 **1.6**	0.592 **0.006**	266.8 **2.0**	569.8 **21.9**	23.3 **0.5**
3	33.9% WPI 32.7% Sorb 14.0% Glyc 19.4% Short	0.507 **0.003**	35.4 **1.0**	0.547 **0.004**	196.5 **2.4**	454.9 **16.3**	27.6 **0.1**
4	33.9% WPI 23.4% Sorb 23.4% Glyc 19.4% Short	0.366 **0.003**	47.1 **2.2**	0.416 **0.001**	384.5 **3.1**	717.8 **44.0**	24.2 **0.5**
5	33.9% WPI 9.3 % Sorb 37.4 % Glyc 19.4% Short	0.186 **0.001**	19.2 **0.1**	0.248 **0.001**	715.4 **0.9**	3625.9 **15.1**	25.3 **1.2**
6	33.9% WPI 46.7% Glyc 19.4% Short	0.091 **0.001**	18.5 **0.4**	0.124 **0.003**	1202.0 **4.0**	6399.6 **162.1**	26.7 **1.3**

[1]%RC = [(Hardness – Initial Hardness) / Initial Hardness] x 100.

[2]$\Delta E = [(a^* - a^*_0)^2 + (b^* - b^*_0)^2 + (L^* - L^*_0)^2]^{1/2}$

MPC and Glycerol. Substituting glycerol for sorbitol syrup had different effects when this was done with a bar based on MPC rather than WPI. Using Bar 2 from Chapter 5 as a reference, substituting 20%, 50%, 80% or 100% glycerol consistently reduced initial bar hardness depending on the glycerol level (Table 6.8). The 100% sorbitol bar had an initial hardness of 65.6 g-force and the 100% glycerol bar had a hardness of only 18.0 g-force. However, the extent of hardening increased with addition of glycerol with the 100% sorbitol bar having a hardness of 513.4 g-force after 43 d at 35°C and the 100% glycerol bar had a hardness of 1251 g-force. So any plasticizing and softening effect provided by glycerol was negated by the promotion of hardening by glycerol.

There was an increase in coloration (or loss of whiteness) of the bars during storage as glycerol was added into the bar formulation, i.e., the ΔE value was higher. The reference bar had ΔE of 14.3 after 43 d storage at 35°C and this progressively increased as more glycerol was added, and the bar with 50% glycerol substitution had ΔE of 20.0. One possible explanation is that the lower viscosity of glycerol increases mobility of the Maillard browning reactants so that lactose can react with amino groups. While this may be the case initially after manufacture when glycerol caused the bars to be softer, the bars with glycerol more rapidly harden and so reactant mobility would decrease. Alternatively, the difference in browning during storage may indicate that the interactions on the surface of the proteins are altered when glycerol is present so that reactions that form Amadori rearrangement during Maillard browning more favorable.

Table 6.8 Effect of substituting glycerol for sorbitol syrup on the mean (SD in bold) water activity (A_w), hardness, relative change in hardness (%RC[1]) and color change (ΔE^2) of high-protein nutrition bar model systems made using milk protein concentrate (MPC) with sorbitol syrup (Sorb) or glycerol (Glyc) and vegetable shortening (Short) based on Bar 2 formulation used in Chapter 5 and described in Table 3.2, during storage at 35°C (n=3).

Test #	Bar Composition	Day 1		Day 43			
		A_w	Hardness (g-force)	A_w	Hardness (g-force)	%RC	ΔE
1	33.9%MPC, 46.7% Sorb 19.4% Short	0.685 **0.001**	65.6 **1.9**	0.703 **0.004**	513.4 **2.7**	683.1 **26.7**	14.4 **0.5**
2	33.9% MPC 37.4 % Sorb 9.3 % Glyc 19.4% Short	0.560 **0.003**	43.4 **0.9**	0.599 **0.610**	557.5 **2.3**	1184.9 **28.1**	15.3 **0.4**
3	33.9% MPC 23.4% Sorb 23.4% Glyc 19.4% Short	0.387 **0.002**	25.0 **1.5**	0.448 **0.003**	784.8 **6.6**	3041.9 **168.1**	20.0 **0.4**
4	33.9% MPC 9.3% Sorb 37.4% Glyc 19.4% Short	0.195 **0.004**	22.0 **0.1**	0.282 **0.003**	988.1 **6.9**	4391.5 **48.0**	22.6 **0.7**
5	33.9% MPC 46.7% Glyc 19.4% Short	0.083 **0.002**	18.0 **0.6**	0.158 **0.003**	1251.0 **6.8**	6853.6 **177.0**	23.3 **0.2**

[1]%RC = [(Hardness – Initial Hardness) / Initial Hardness] x 100

[2]$\Delta E = [(a^* - a^*_0)^2 + (b^* - b^*_0)^2 + (L^* - L^*_0)^2]^{1/2}$

Combining WPI and MPC with Sorbitol and Glycerol

A series of experiments in which WPI and MPC were used together in bar formulations, along with substitution of 20% of the sorbitol syrup with glycerol were conducted. These bars were made in which total protein powder was 33.9% of the bar formulation (as described in Chapter 5), and then in formulations containing 36%, 38% and 40% protein powder. With an increase in protein powder to 36%, sorbitol syrup/glycerol component was reduced to 44.6% and the shortening to 17.4%. With further increase in protein the sorbitol syrup/glycerol component was maintained at 44.6% and the shortening decreases to 17.4% and then 15.4%., the amount of shortening was reduced from 19.4% to 15.4%.

As protein content was increased, the initial hardness of the bars increased from 36.5 g-force for the reference 100% WPI bar with 33.9% protein to 54.4, 101 and 175 g-force for the bars with 36%, 38% and 40% protein powder respectively. This increase in hardness was also apparent after 43 d storage at 35°C with the bars having hardness values of 398, 601, 859 and 1230 g-force respectively. This demonstrates the challenge that is faced in attempting to increase the amount of protein in HPN bars. As the protein level is increased the bars become excessively hard during storage and the rate of hardening increases, although this is not apparent when calculated as the percent changes based upon the initial hardness of the bars.

When MPC was added into a 33.6% protein powder bar formulation based on WPI along with a 20% substitution of glycerol for sorbitol syrup, the initial bar hardness decreased slightly with 20% MPC added and then slightly increased (Table 6.9). The initial bar hardness was 27.3 g-force compared to 36.5 g-force for the reference bar made

Table 6.9 Bar characteristics (means, SD in bold) of bar model systems made using 33.9% protein powder (100:0, 80:20, 50:50 and 20:80 mixtures of whey protein isolate (WPI) and milk protein concentrate (MPC)) with (80:20) sorbitol syrup (Sorb) and glycerol (Glyc), and vegetable shortening (Short) based on Bar 1 formulation used in Chapter 5 and described in Table 3.2, during storage at 35°C (n=3).

Test #	Bar Composition	Day 1		Day 43			
		A_w	Hardness (g-force)	A_w	Hardness (g-force)	%RC[1]	ΔE[2]
1	33.9% WPI 46.7% Sorb 19.4% Short	0.633 **0.003**	36.5 **1.0**	0.661 **0.003**	398.3 **5.3**	992.8 **35.5**	20.7 **0.4**
2	27.1%WPI, 6.8% MPC 37.4% Sorb 9.3% Glyc 19.4% Short	0.540 **0.004**	27.3 **0.9**	0.578 **0.600**	207.5 **3.0**	661.4 **13.4**	20.3 **1.1**
3	17.0% WPI 17.0% MPC 37.4% Sorb 9.3% Glyc 19.4% Short	0.544 **0.005**	25.4 **1.3**	0.576 **0.003**	55.4 **2.3**	118.4 **12.1**	18.5 **0.6**
4	6.8%WPI, 27.1% MPC 37.4%Sorb, 9.3%Glyc 19.4% Short	0.550 **0.006**	32.0 **1.0**	0.586 **0.003**	289.5 **1.4**	804.4 **24.1**	19.2 **0.1**

[1]%RC = [(Hardness – Initial Hardness) / Initial Hardness] x 100

[2]$\Delta E = [(a^* - a^*_0)^2 + (b^* - b^*_0)^2 + (L^* - L^*_0)^2]^{1/2}$

using 100% WPI. The extent of hardening was also reduced and after 43 d storage at 35°C, the 20% MPC bar had a hardness of 207.5 g-force compared to 398.3 g-force for the reference bar. When 50% MPC was used, bar hardening was extensively reduced and after 43 d this bar was still soft and malleable with a hardness of only 55.4 g-force (Table 6.9). However, when more MPC was added into the mix (20:80 WPI:MPC), bar hardening during storage returned to its characteristic level similar to using 100% WPI and after 43 d had a hardness of 289.5 g-force.

Since combining WPI and MPC in a 50:50 ratio minimized bar hardening during storage (when 20% of the sorbitol syrup was also substituted with glycerol) it was of interest to see how high a protein level could be used in a HPN bar system. Adding the glycerol into a 36% protein powder bar resulted in a small reduction in hardening after 43 d at 35° from 601.9 g-force for the reference bar (containing WPI, sorbitol syrup and shortening) to 462.3 g-force. However, when the 50:50 WPI:MPC powder blend was used the d-43 bar hardness was only 95.1 g-force.

The same effect was observed with a 38% protein powder bar system (Table 6.10) in which the d-43 hardness for the reference bar was 859 g-force, the hardness for the glycerol-added bar was 619 g-force, while adding glycerol and MPC resulted in a d-43 hardness of only 153.9 g-force (Table 6.11). And for 40% protein powder bars (Table 6.2), the reference bar had a 43-d hardness of 1,230 g-force, while the hardness of the bar made with added glycerol and MPC was only 335 g-force.

Table 6.10 Bar characteristics (means, SD in bold) of bar model systems made using 36% protein powder (50:50 mixture of whey protein isolate (WPI) and milk protein concentrate (MPC)) with (80:20) sorbitol syrup (Sorb) and glycerol (Glyc), and vegetable shortening (Short) during storage at 35°C (n=3).

Test #	Bar Composition	Day 1		Day 43			
		A_w	Hardness (g-force)	A_w	Hardness (g-force)	%RC[1]	ΔE[2]
1	36.0% WPI 44.6% Sorb 17.4% Short	0.638 **0.002**	54.4 **2.3**	0.659 **0.003**	601.9 **9.0**	1007.4 **36.0**	25.3 **0.2**
2	36.0% WPI 35.7% Sorb 8.9% Glyc 17.4% Short	0.543 **0.004**	46.5 **2.4**	0.575 **0.6**	462.3 **3.8**	896.1 **55.0**	27.7 **0.2**
3	18.0% WPI 18.0%MPC, 35.7% Sorb 8.9% Glyc 17.4% Short	0.566 **0.003**	22.2 **2.0**	0.620 **0.01**	95.1 **1.2**	328.4 **33.0**	21.7 **0.2**

[1]%RC = [(Hardness – Initial Hardness) / Initial Hardness] x 100.

[2]$\Delta E = [(a^* - a^*_0)^2 + (b^* - b^*_0)^2 + (L^* - L^*_0)^2]^{½}$

Table 6.11 Bar characteristics (means, SD in bold) of bar model systems made using 38% protein powder (50:50 mixture of whey protein isolate (WPI) and milk protein concentrate (MPC)) with (80:20) sorbitol syrup (Sorb) and glycerol (Glyc), and vegetable shortening (Short) during storage at 35°C (n=3).

Test #	Bar Composition	Day 1		Day 43			
		A_w	Hardness (g-force)	A_w	Hardness (g-force)	%RC[1]	ΔE[2]
1	38.0% WPI 44.6%Sorb, 17.4% Short	0.634 **0.002**	101.0 **4.3**	0.656 **0.003**	859.0 **5.0**	751.5 **37.7**	27.5 **0.5**
2	38.0% WPI 35.7% Sorb 8.9% Glyc 17.4% Short	0.544 **0.006**	56.2 **1.6**	0.580 **0.583**	619.0 **7.4**	1002.2 **43.9**	28.2 **0.7**
3	19.0% WPI 19.0%MPC, 35.7% Sorb 8.9% Glyc 17.4% Short	0.554 **0.002**	33.6 **1.5**	0.594 **0.005**	153.9 **3.1**	357.8 **10.8**	21.5 **0.3**

[1]%RC = [(Hardness – Initial Hardness) / Initial Hardness] x 100.

[2]$\Delta E = [(a^* - a^*_0)^2 + (b^* - b^*_0)^2 + (L^* - L^*_0)^2]^{½}$

Table 6.12 Bar characteristics (means, SD in bold) of bar model systems made using 40% protein powder (50:50 mixture of whey protein isolate (WPI) and milk protein concentrate (MPC)) with (80:20) sorbitol syrup (Sorb) and glycerol (Glyc), and vegetable shortening (Short) during storage at 35°C (n=3).

Test #	Bar Composition	Day 1		Day 43			
		A_w	Hardness (g-force)	A_w	Hardness (g-force)	RC[1]%	ΔE^2
1	40.0%WPI, 44.6%Sorb 15.4% Short	0.618 **0.002**	175.1 **3.2**	0.663 **0.001**	1230.2 **6.1**	602.6 **12.8**	26.9 **0.5**
2	40.0% WPI 35.7% Sorb 8.9% Glyc 15.4% Short	0.550 **0.005**	85.3 **3.0**	0.586 **0.583**	860 **8.5**	908.2 **7.0**	30.6 **2.0**
3	20%WPI, 20%MPC, 35.7% Sorb 8.9% Glyc 15.4% Short	0.547 **0.005**	44.9 **0.8**	0.584 **0.004**	335.1 **3.7**	646.4 **5.0**	23.2 **0.4**

[1]%RC = [(Hardness – Initial Hardness) / Initial Hardness] x 100

[2]$\Delta E = [(a^* - a^*_0)^2 + (b^* - b^*_0)^2 + (L^* - L^*_0)^2]^{½}$

Disulfide Bonding

To test if disulfide bonds between proteins were forming during storage, bars were made with the addition of DTT as a reducing agent to prevent formation of disulfide bonds. These bars were then stored at 35°C for 43 d and compared to a similar bar in which no DTT had been added. No differences were observed in either initial hardness or final hardness as a result of adding DTT, when used in conjunction with glycerol or sorbitol syrup (Table 6.13).

Table 6.13 Effect of 5 mM dithiothreitol (DTT) on water activity (A_w), hardness, relative change in hardness (%RC[1]) and color change (ΔE^2) of high-protein nutrition bar model systems made using whey protein isolate (WPI) with sorbitol syrup (Sorb) or glycerol (Glyc) and vegetable shortening (Short) based on Bar 1 and 3 formulation used in Chapter 5 and described in Table 3.2, during storage at 35°C (n=3).

Test #	Bar Composition	Day 1		Day 43			
		A_w	Hardness (g-force)	A_w	Hardness (g-force)	%RC	ΔE
1	33.9% WPI 46.7% Sorb 19.4% Short	0.633 **0.002**	31.7 **1.7**	0.648 **0.003**	318.6 **6.3**	906.8 **52.9**	17.6 **0.32**
2	33.9% WPI 46.7%Sorb, 19.4% Short 5.6 µl DTT	0.635 **0.004**	29.4 **1.0**	0.644 **0.003**	310.6 **10.5**	956.4 **17.1**	17.7 **0.8**
3	33.9% WPI 46.7% Sorb 19.4% Short 25 µl DTT	0.638 **0.003**	30.4 **1.1**	0.651 **0.004**	294.9 **6.4**	870.2 **54.9**	17.1 **0.6**
4	33.9%WPI, 46.7% Glyc 19.4% Short	0.118 **0.003**	16.3 **1.0**	0.149 **0.005**	1217.2 **14.3**	7385.6 **469.2**	24.4 **0.4**
5	33.9% WPI 46.7 % Glyc 19.4% Short 5.6 µl DTT	0.116 **0.003**	16.6 **0.8**	0.144 **0.004**	1221.6 **16.0**	7282.0 **262.6**	25.1 **0.3**
6	33.9% WPI 46.7% Glyc 19.4% Short 25 µl DTT	0.112 **0.004**	16.3 **1.0**	0.135 **0.004**	1274.7 **13.8**	7739.7 **483.2**	25.3 **1.3**

[1]%RC = [(Hardness – Initial Hardness) / Initial Hardness] x 100

[2]$\Delta E = [(a^* - a^*_0)^2 + (b^* - b^*_0)^2 + (L^* - L^*_0)^2]^{1/2}$

SUMMARY

Any differences in hardening related to using MPC instead of WPI were not related to MPC having higher lactose content. Adding lactose does lower the protein content of HPN bars and therefore makes the bars initially softer, but does not slow down the rate of hardening. Increasing the lactose content increases the rate of browning, so having less browning in bars containing MPC rather than WPI is related to the proteins and not the lactose. It suggests there are more available amino groups in WPI than MPC.

Adding the polysorban surfactant Tween 20 increased hardness in bars made using sorbitol but decreased hardness in bars made using glycerol. Tween 20 had no effect on hardening during storage. The differences between bars made using glycerol or sorbitol syrup, may be related to the relative size of Tween 20 to these polyol cosolvents.

Chelating some of the calcium by adding sodium citrate to bars made using MPC made bars initially harder and had negligible effect on retarding bar hardening.

Using liquid oil rather than shortening had no effect on hardening in bars made with sorbitol syrup. In contrast, using liquid oil increased hardening in bars made using glycerol. This indicates that the lipid in HPN bars made using sorbitol acts as an inactive filler. In contrast, when glycerol is the solvent, the presence of a more mobile lipid fraction enhances protein-protein aggregation.

If the lipid is oxidized to generate sites that can interact electrostatically, this promotes hardening in bars made using glycerol. In this case, the oxidized oil molecules are promoting unfavorable interactions (i.e., interactions that promote protein-protein aggregation) between glycerol and surface moieties of the proteins.

Mixing glycerol with sorbitol can either accelerate or retard hardening in HPN bars made using WPI, depending on the amount of glycerol added. If [glycerol] > [sorbitol] then hardening was accelerated. If [glycerol] < [sorbitol] then hardening was retarded. This suggests that glycerol interacts with the protein in different ways. At lower concentration compared to sorbitol, glycerol interacts with the protein in ways that retards protein-protein aggregation.

When glycerol was added to bars made using MPC, rate of hardening increased at all levels. Having the protein powder in MPC bars still in particulate form, apparently causes different interactions than occurs with the more dispersed proteins from WPI.

As protein level increases the bars are harder, whether MPC or WPI was used. When both a mixture of 50:50 WPI and MPC, and an 80:20 mixture of sorbitol syrup and glycerol were included in HPN bar formulations, the hardening of the bars during storage was further retarded. This allows HPN bars to be made that contain higher protein levels.

Recommendations for Future Research. From this collection of studies, the most effective way to retard hardening of HPN bars appears to be:

1. Substituting MPC for 50% of the WPI so that the particulate protein powder particles of MPC interrupt the formation of an extensive protein network structure, and
2. Adding a small portion of glycerol into the bar so that there is a three-component cosolvent mixture that contains cosolvents of different sizes.

Hardening of HPN bars by protein aggregation appears to be related to how the cosolvents interact with polar and hydrophobic moieties on the protein surface, and does not appear to involve disulfide bond formation between proteins.

CHAPTER 7.

PHASE 3: IMPACT OF MULTI-SIZED COSOLVENTS AND MIXED MILK AND WHEY PROTEINS ON PREVENTION OF PROTEIN AGGREGATION AND HARDENING OF HPN BARS.

INTRODUCTION

From work in Phase 1 and 2 of this research, having a mixture of cosolvents in the bar formulation (i.e., substituting part of the sorbitol syrup with glycerol) has the most impact on retarding hardening of HPN bars during storage. In contrast, using only glycerol accelerates hardening. This suggests that glycerol interacts differently with the surface of the protein when it is combined with the sorbitol syrup, and produces a system of cosolvents that function different because of their size differences.

Having a combination of MPC and WPI as protein powders in HPN bars also retards hardening. Previous work by McMahon and others (2009) had demonstrated that adding partially hydrolyzed whey proteins helps to soften bars, but bars made with intact whey proteins (as in WPI) harden during storage. However, using MPC on its own does not produce a satisfactory HPN bar. Such bars are brittle and hard with a coarse texture.

This Phase 3 portion of the research was designed as a 2 x 2 factorial experiment. It compares a reference HPN bar (made using WPI, sorbitol syrup and shortening) with bars in which half of the WPI powder was replaced with MPC, and 20% of the sorbitol syrup was replaced with glycerol. The aim was to elucidate the chemical mechanism by which bar hardening can be inhibited, so that bars will stay taffy-like during storage without becoming hard and brittle.

Of the various factors that had been suggested in the literature on HPN bars, it had been observed during Phase 2 and 3 research that:

1. Some moisture migration occurs that involves intermediate water moving into the bound water state and some becoming bulk water. It is important to remember that in HPN bars, 99% of the water exists as bound water. Such water molecules are strongly interacting with the cosolvents. This makes it impossible to model HPN bars using buffer systems in which more water exists in the bulk or intermediate state. Sorbitol syrup contains virtually no bulk or even intermediate water (see Appendix A, Figure A.3). This suggests that movement of water only involves water molecules that are interacting with the proteins.
2. Protein aggregation does occur during bar storage and an extensive protein network, in conjunction with the cosolvent system, is formed. The proteins of MPC appear to mainly stay within the powder particles, with some of the more soluble proteins (possibly the whey proteins) being distributed throughout the sorbitol syrup.
3. Protein aggregation does not appear to be induced because of a macroconstituent phase separation between the water, cosolvent and protein portions of the HPN bar matrix. No phase separation was observed in bars examined during Phase 1. What had previously been reported by McMahon and others (2009) as evidence of phase separation, was determined to be air bubbles and voids within the bars.
4. Protein aggregation was not a result of Maillard browning reactions between protein amino groups and reducing sugars. There was minimal browning during

storage of bars in Phase 1, and no practical difference in color of HPN bars based upon their extent of hardening during storage.

5. Protein aggregation does not appear to involve disulfide bond formation between the proteins. Adding DTT to keep cysteine sulfur groups in the reduced form, did not prevent hardening.
6. No glass transitions were observed within the temperature range at which the HPN bars were stored. No crystallization of sorbitol was observed. Therefore, these type of changes in the carbohydrate portion of the HPN bars do not appear to be involved in bar hardening during storage.
7. Calcium does not appear to be involved in the protein aggregation during storage. Adding a calcium chelating agent did not retard hardening.

RESULTS

Bar Color

Initially after manufacture, the bars were similar in appearance to those made during the Phase 1 experiments, and were white to creamy in color. Adding MPC as part of the protein portion significantly ($P < 0.001$) affected WI of the bars as well as L*, a* and b* values (Table 7.1). Adding glycerol to the solvent/cosolvent mix had no impact (P =0.32) on overall whiteness, although it did affect the a* value ($P = 0.042$) (Table 7.2). Storage time influenced bar color, including ΔE during the 84 d storage at 35°C. There were also some significant interactions of carbohydrate x protein, carbohydrate x time, and protein x time for L*a*b* values, as well as for WI and ΔE.

Table 7.1 Analysis of variance of color of bars made using whey protein isolate and milk protein concentrate with different combinations of sorbitol syrup (SS) and glycerol as measured using L*a*b* color system.

		P				
Effect	**df**	**L***	**a***	**b***	**WI[1]**	**ΔE[2]**
Carbohydrate (Carb)	1	0.2685	0.0419	0.8149	0.3237	0.6972
Protein (Prot)	1	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Carb x Prot	1	0.1230	0.0286	0.0081	0.2106	0.0590
Time (T)	6	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Carb x T	6	0.1795	0.1770	0.0016	0.0038	0.0001
Prot x T	6	<0.0001	0.0018	<0.0001	<0.0001	<0.0001
Carb x Prot x T	6	<0.0001	0.1495	0.0312	0.0009	0.0016

[1]WI = 100- $[(100\text{-}L^*)^2 + (a^*)^2 + (b^*)^2]^{½}$

[2]$\Delta E = [(a^* - a^*_0)^2 + (b^* - b^*_0)^2 + (L^* - L^*_0)^2]^{½}$

Table 7.2 Color of bars made using whey protein isolate (WPI) and milk protein concentrate (MPC) with different combinations of sorbitol syrup (SS), and glycerol (GLYC) as measured using L*a*b* color system and whiteness index (WI[1]) on d 1 after manufacture.

	Sorbitol		**Sorbitol+Glycerol**	
Color value	**WPI**	**WPI/MPC**	**WPI**	**WPI/MPC**
L*	89.43^{c}	91.77^{a}	89.81^{bc}	90.51^{b}
a*	0.45^{hij}	0.01^{l}	0.36^{ijk}	0.07^{kl}
b*	11.14^{m}	10.56^{mn}	10.20^{n}	10.27^{mn}
WI[1]	84.6^{c}	86.6^{a}	85.6^{b}	86.0^{ab}

[1]WI = 100- $[(100\text{-}L^*)^2 + (a^*)^2 + (b^*)^2]$ ½

The whitest bar was Bar B containing WPI/MPC and sorbitol syrup, both in terms of having the highest L* value, low a* and b* values and the highest overall whiteness. Bar B had WI of 91.8. The bar with the lowest whiteness was Bar A with WI of 89.4 (Table 7.2). The increased whiteness of the bars containing MPC was probably related to increased light scattering from having particles of powder still present. With bars made using WPI, the protein appeared more disperse. Initial mean WI for the bars was in the range 84.6 to 86.6, with Bar B > Bar D > Bar C > Bar A.

During storage (Figure 7.1), all of the bars became darker in color with significant changes occurring within the first 7 d storage at 35°C. The WI of the bars at d 7 had decreased by 10 units for the WPI bars (Bar A and C), and only by 4 units in the WPI/MPC bars (Bar B and D) (Table 7.3). The change in color continued through

Table 7.3 Change in color based on Whiteness Index (WI[1]) and Total Color Change (ΔE^2) values of high-protein nutrition bars made using whey protein isolate (WPI) and milk protein concentrate (MPC) with sorbitol syrup or sorbitol syrup/glycerol (as in Table 3.2) during 84 day of storage at 35°C.

Storage Time (d)	Color							
	Sorbitol				Sorbitol+Glycerol			
	WPI (Bar A)		WPI/MPC (Bar B)		WPI (Bar C)		WPI/MPC (Bar D)	
	WI	ΔE	WI	ΔE	WI	ΔE	WI	ΔE
1	84.6^{c}	0.0^{M}	86.6^{a}	0.0^{M}	85.6^{b}	0.0^{M}	86.0^{ab}	0.0^{M}
7	74.1^{hi}	10.8^{GH}	82.5^{d}	4.6^{KL}	75.3^{g}	10.4^{H}	82.4^{d}	3.8^{L}
14	72.1^{j}	12.6^{F}	80.2^{e}	6.6^{J}	73.4^{i}	12.3^{F}	81.0^{e}	5.1^{K}
21	70.7^{kl}	14.0^{E}	78.5^{f}	8.2^{I}	69.9^{l}	15.8^{D}	79.3^{f}	6.8^{J}
42	68.8^{m}	15.9^{D}	74.7^{gh}	11.8^{FG}	67.2^{n}	18.5^{C}	74.7^{gh}	11.7^{FG}
63	65.0^{o}	19.7^{B}	71.2^{k}	15.5^{D}	65.3^{o}	20.6^{B}	70.2^{l}	16.5^{D}
84	61.0^{p}	23.7^{A}	66.3^{n}	20.5^{B}	61.9^{p}	23.8^{A}	66.4^{n}	20.2^{B}

[1]WI = 100- $[(100-L^*)^2 + (a^*)^2 + (b^*)^2]$ ½

[2]$\Delta E = [(a^* - a^*0)^2 + (b^* - b^* 0)^2 + (L^* - L^*0)^2]$ ½.

$^{abc\ldots nop}$WI means with same letter were not significantly different, α = 0.05.

$^{ABC\ldots.KLM}\Delta E$ means with same letter were not significantly different, α = 0.05.

storage. A significant change in WI occurred every week. The L* values decreased (see Appendix B, Table B.1) as a* and b* values increased (Appendix B, Table B.2 and B.3). After 84 d storage, the mean WI for the bars was in the range 61.0 to 66.4 with Bar D > Bar B > Bar C > Bar A. The ΔE after 84 d was in the range of 20.2 to 23.8 units, with less change occurring in the bars with added MPC. By visual appearance (Figure 7.1), after 84 d the extent of browning was Bar A > Bar C > Bar D > Bar B.

The extent of browning of all the bars was still fairly minimal compared to HPN bars made using reducing sugars such as fructose, glucose and maltose. As shown by McMahon and others (2009), such bars lose far more whiteness and develop a distinct brown color, or even become black if HWPI is used to retard bar hardening. As discussed below, there was little relationship between extent of browning and bar hardening during storage.

Water Activity

Water activity of the HPN bars was significantly influenced ($P < 0.0001$) by both the inclusion of MPC and glycerol in the bar formulation (Table 7.4). There was also a significant carbohydrate x protein interaction ($P < 0.004$). After manufacture (d 1) there were significant differences ($P < 0.05$) in water activity. Bars A, B, C and D had water activity of 0.626, 0.650, 0.571, and 0.578, respectively (Table 7.5). As discussed in Chapter 5, MPC has a slightly higher moisture content than WPI. This was attributed to being the cause for the slight increase in water activity of Bar B compared to Bar A. Replacing some of the sorbitol syrup with glycerol further decreased water activity as water in the syrup was replaced with glycerol.

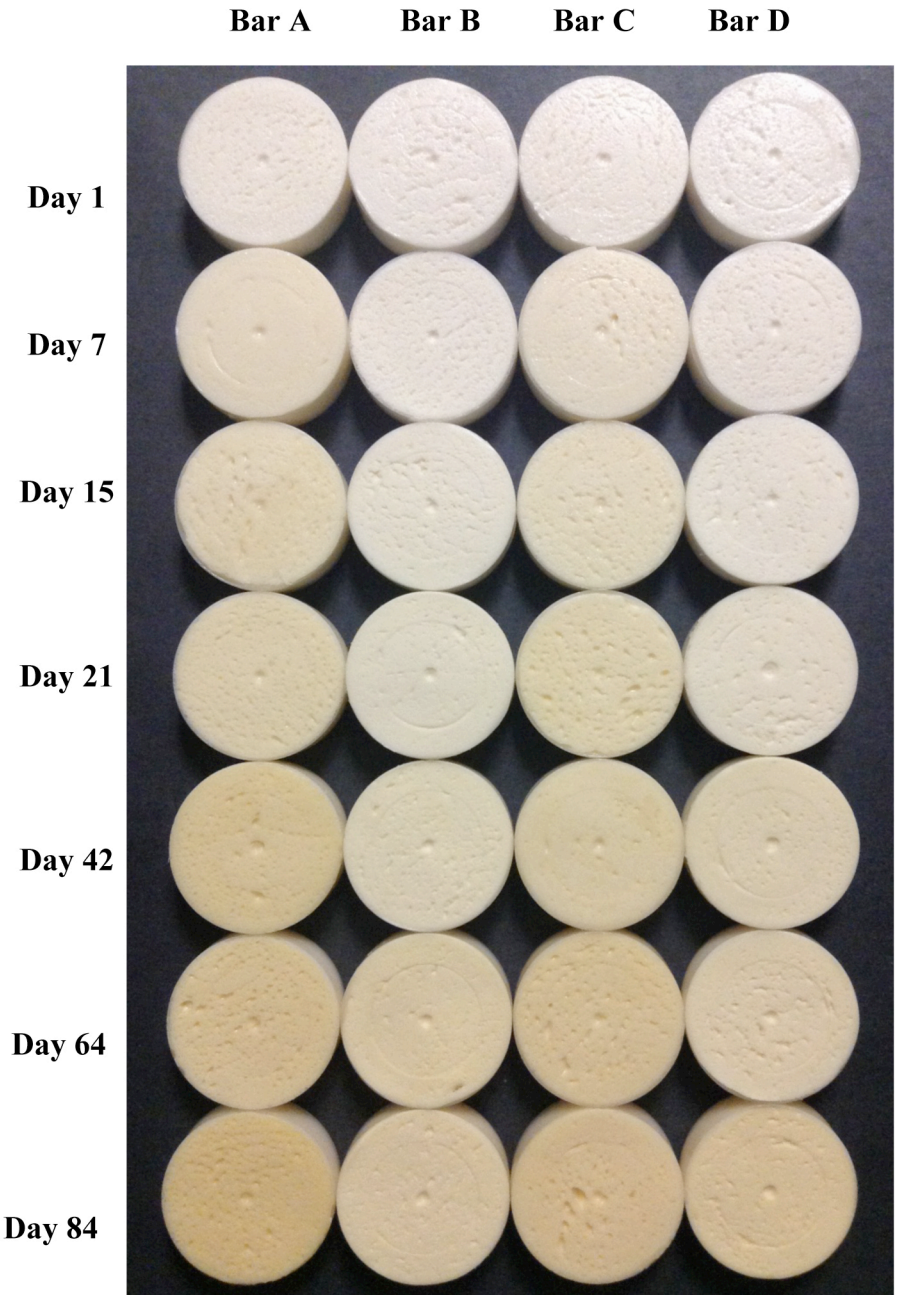

Figure 7.1 High-protein nutrition bars made with shortening showing (A) the Control bar made using whey protein isolate (WPI) and sorbitol syrup, compared to (B) with 50% of WPI replaced with milk protein concentrate (MPC), (C) with 20% of the sorbitol syrup replaced with glycerol, and (D) with both the MPC and glycerol substitution (as described in Table 3.2) during 84 d storage at 35°C.

Table 7.4 Analysis of variance of water activity (Aw), hardness and percent relative change in hardness during storage (%RC[1]) of bars made using whey protein isolate and milk protein concentrate with different combinations of sorbitol syrup and glycerol during 84 d storage at 35°C.

Effect	*P*			
	df	A_w	Hardness	%RC
Carbohydrate (Carb)	1	<0.0001	<0.0001	<0.0001
Protein (Prot)	1	<0.0001	<0.0001	0.0001
Carb x Prot	1	0.0004	0.0072	<0.0001
Time (T)	6	<0.0001	<0.0001	<0.0001
Carb x T	6	<0.0001	<0.0001	<0.0001
Prot x T	6	0.6277	<0.0001	<0.0001
Carb x Prot x T	6	0.6124	<0.0001	<0.0001

[1]%RC = [(Hardness – Hardness at d 1) / Hardness at d 1] x 100.

Table 7.5 Water activity of high-protein nutrition bars made using whey protein isolate (WPI) or a 50:50 WPI/milk protein concentrate (MPC) mixture, combined with shortening, sorbitol syrup, and glycerol (as described in Table 3.2) during 84 d of storage at 35°C.

	Water Activity			
Storage Time (d)	Sorbitol		Sorbitol+Glycerol	
	WPI (Bar A)	WPI/MPC (Bar B)	WPI (Bar C)	WPI/MPC (Bar D)
1	0.626^{f}	0.650^{e}	0.571^{l}	0.578^{k}
7	0.650^{e}	0.670^{c}	0.580^{jk}	0.589^{ghi}
14	0.660^{d}	0.679^{b}	0.583^{ijk}	0.591^{g}
21	0.663^{d}	0.682^{b}	0.584^{ijk}	0.591^{gh}
42	0.665^{cd}	0.681^{b}	0.584^{ijk}	0.592^{g}
63	0.669^{c}	0.682^{b}	0.585^{hij}	0.592^{g}
84	0.669^{c}	0.689^{a}	0.582^{jk}	0.589^{ghi}

[abc…ijk]means with same letter were not significantly different, α = 0.05.

There was a significant increase in water activity during the first week of storage of about 0.02 for Bars A and B, and about 0.01 for Bars C and D that contained glycerol. This increase in water activity was assumed related to further solvation of the protein powder, with the water in those powders becoming part of the overall solvent/cosolvent phase. With continued storage, the water activity in Bars A and B continued to increase, but at a slower rate. After 84 d, the water activity of Bars A and B was 0.669 and 0.689, respectively. There was no further increase in water activity in the bars containing glycerol (Bars C and D) after 7 d. After 84 d, these bars had water activity of 0.582 and 0.589, respectively (Table 7.5).

Hardness

Hardness of the bars was significantly influenced ($P < 0.001$) by adding MPC and glycerol into the bar formulations, as well as by a protein x carbohydrate interaction ($P = 0.007$) (Table 7.4). Storage time at 35°C, and interactions of time with protein and carbohydrate were also significant ($P < 0.001$). When measured on d 1 after manufacture, addition of glycerol into the formulation slightly increased bar hardness ($P < 0.05$), with Bar C having a hardness of 481 g-force compared to 349 g-force for Bar A (Table 7.6). Substituting half of the WPI with MPC caused a very large decrease in bar hardness[1], with Bars B and D having d-1 hardness of only 50 and 54 g-force, respectively. Having a combination of WPI and MPC produced softer bars that were not as crumbly and brittle as the bars made using MPC only (see Chapter 5).

[1]A different probe (1-cm long with square tip) and only 7-mm penetration was used for testing hardness of Bars A, B, C and D, so hardness values were different from Bars 1, 2, 3 and 4 when a longer by chisel-point probe was used for measuring hardness.

Table 7.6 Hardness of high-protein nutrition bars made using whey protein isolate (WPI) or a 50:50 WPI/milk protein concentrate (MPC) mixture, combined with shortening, sorbitol syrup, and glycerol (as described in Table 3.2) during 84 d of storage at 35°C.

Storage Time (d)	Hardness (g-force)			
	Sorbitol		Sorbitol+Glycerol	
	WPI (Bar A)	WPI/MPC (Bar B)	WPI (Bar C)	WPI/MPC (Bar D)
1	349.1^{m}	50.1^{z}	481.3^{l}	53.8^{y}
7	676.2^{ef}	114.0^{v}	512.2^{k}	80.4^{x}
14	691.2^{e}	142.4^{u}	528.3^{j}	99.5^{w}
21	752.5^{d}	177.3^{r}	554.8^{i}	112.9^{v}
42	832.7^{c}	216.6^{p}	585.7^{h}	148.8^{t}
63	879.0^{b}	243.2^{o}	601.7^{g}	161.3^{s}
84	1000.7^{a}	269.5^{n}	661.3^{f}	194.4^{q}

$^{abc\ldots xyz}$ means with same letter were not significantly different, $\alpha = 0.05$.

During storage at 35°C, the bars underwent hardening that commenced within the first week (Table 7.6). The bars made with only sorbitol syrup as the solvent/cosolvent (Bars A and B), hardened very rapidly and had d-7 hardness values of 676 and 115 g-force, respectively. Smaller increases in hardness occurred in Bars C and D. When compared to the initial hardness of the bars, Bars A and B had increased in hardness by 94% and 128%, respectively, by d 7, while Bars C and D increased by only 6.5% and 53%, respectively (Table 7.7). These changes in hardness continued through storage and after 84 d, Bar A had increased almost 2-fold, Bar B over 4-fold, Bar C only by one-third, and Bar D by 2.5-fold. These bars had %RC of 187%, 438%, 37%, and 262% (Table 7.7), and bar hardness of 1000, 270, 661 and 194 g-force, respectively. Although Bar C had the lowest %RC during storage, Bar D had the lowest hardness at the end of the 84 d storage. This formulation, containing both WPI and MPC as well as sorbitol syrup and glycerol, was considered the most effective formulation for minimizing bar hardening.

Table 7.7 Increase in hardness expressed as percent relative hardness (%RC[1]) of high-protein nutrition bars made using whey protein isolate (WPI) or a 50:50 WPI/milk protein concentrate (MPC) mixture, combined with shortening, sorbitol syrup, and glycerol (as described in Table 3.2) during 84 d of storage at 35°C.

Storage Time (d)	%RC			
	Sorbitol		Sorbitol+Glycerol	
	WPI (Bar A)	WPI/MPC (Bar B)	WPI (Bar C)	WPI/MPC (Bar D)
7	93.7^{m}	127.5^{k}	6.5^{tu}	53.0^{o}
14	98.0^{m}	183.9^{gh}	9.8^{st}	85.1^{n}
21	115.6^{l}	253.7^{e}	15.3^{rs}	110.0^{l}
42	138.5^{j}	332.1^{c}	21.7^{qr}	176.7^{h}
63	151.8^{i}	385.1^{b}	25.0^{q}	199.9^{f}
84	186.6^{g}	437.6^{a}	37.4^{p}	261.8^{d}

[1]%RC = [(Hardness – Hardness at d 1) / Hardness at d 1] x 100

$^{abc\ldots stu}$means with same letter were not significantly different, $\alpha = 0.05$.

Bulk, Intermediate and Bound Water

The amount of bulk and intermediate water in the HPN bars was determined based on latent heat of fusion of water, combined with endothermic transitions between about -20°C and -5°C related to melting of intermediate (weakly interacting) water and that between about -2°C and 5°C of bulk water. An example of a DSC thermogram is shown in Figure 7.2.

Intermediate Water. The onset temperature for melting of intermediate water was significantly influenced ($P < 0.001$) by adding MPC into the bar formulation, and by adding glycerol (Table 7.8). Storage time was also a significant effect as well as the various two-way interactions. Use of glycerol, and storage time, influenced peak temperature for intermediate water. Protein was not a significant main effect (Appendix B, Table B.5). The control formulation (Bar A), had an intermediate water transition onset

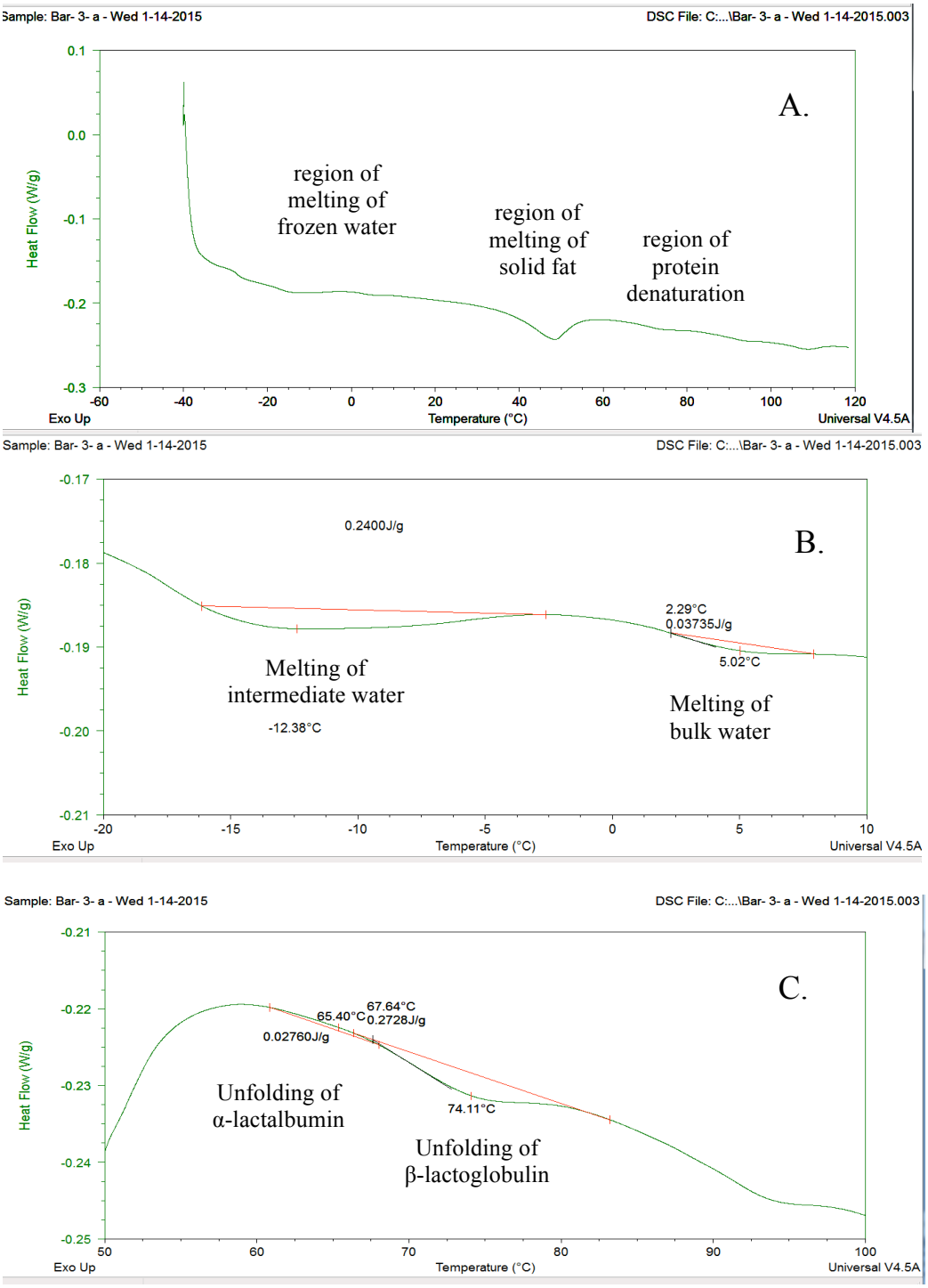

Figure 7.2 Typical thermogram for high-protein nutrition bars showing (A) thermal transition from -40°C to 120°C, and (B) from -20°C to 20°C from which the onset and peak temperatures and enthalpic change (J/g) were calculated for intermediate and bulk water, and (C) from 40°C to 120°C from onset and peak temperatures and enthalpic change were calculated for transitions attributed to denaturation of α-lactalbumin and β-lactoglobulin.

Table 7.8 Analysis of variance of intermediate, bulk and bound water of high-protein nutrition bars made using whey protein isolate and milk protein concentrate with different combinations of sorbitol syrup and glycerol during 84 d storage at 35°C.

Effect	*P*			
	df	Intermediate water	Bulk water	Bound water
Carbohydrate (Carb)	1	<0.0001	<0.0001	<0.0001
Protein (Prot)	1	<0.0001	0.0001	<0.0001
Carb x Prot	1	<0.0001	0.5983	<0.0001
Time (T)	4	<0.0001	<0.0001	<0.0001
Carb x T	4	0.0060	0.0399	0.0032
Prot x T	4	0.0122	0.0214	0.0372
Carb x Prot x T	4	<0.0001	0.1172	0.0001

temperature of -13.0°C. Adding glycerol lowered ($P < 0.05$) the onset temperature to -14.0°C, while adding MPC lowered the onset temperature to -16.2 and -15.8°C for Bars B and D, respectively (Table 7.9). The peak temperatures were similar and was -12.2°C for Bar A and D which was significantly lower than for Bar B (-10.7°C) and Bar C (-11.1°C). Melting of intermediate water at a slightly lower temperature, suggests a slight increase in extent of weak interactions between the water molecules and other components in the bars.

The amount of intermediate, bulk and bound water in the bars was influenced ($P < 0.0001$) by addition of either MPC or glycerol (Table 7.8). For intermediate and bound water, there was a significant carbohydrate x protein interaction. There were significantly different ($P < 0.05$) amounts of intermediate water in the bars after manufacture, even though the bars had intermediate water levels of only 0.066, 0.101, 0.079 and 0.069 g/100 g solids for Bars A, B, C and D respectively (Table 7.9). This

Table 7.9 Onset and peak temperature for melting of intermediate water and calculated quantity of intermediate water in high-protein nutrition bars of high-protein nutrition bars made using whey protein isolate (WPI) or a 50:50 WPI/milk protein concentrate (MPC) mixture, combined with shortening, and sorbitol syrup and glycerol (as described in Table 3.2) during 84 day of storage at 35°C.

Storage time (d)	Bar		Intermediate Water		
			Onset Temp (°C)	Peak Temp (°C)	Quantity (g/100 g solids)
1	Sorbitol	WPI	-13.00^{a}	-12.15^{klm}	0.0660^{hij}
		WPI/MPC	-16.20^{h}	-10.86^{ij}	0.1009^{a}
	Sorbitol + Glycerol	WPI	-14.00^{c}	-11.05^{j}	0.0793^{e}
		WPI/MPC	-15.78^{gh}	-12.15^{klm}	0.0689^{fg}
21	Sorbitol	WPI	-13.59^{bc}	-11.15^{j}	0.0647^{j}
		WPI/MPC	-16.10^{h}	-12.52^{m}	0.0988^{ab}
	Sorbitol + Glycerol	WPI	-14.83^{d}	-11.20^{j}	0.0701^{f}
		WPI/MPC	-15.07^{de}	-10.26^{fg}	0.0677^{fghi}
42	Sorbitol	WPI	-14.00^{c}	-9.31^{c}	0.0649^{ij}
		WPI/MPC	-15.17^{def}	-12.40^{lm}	0.0973^{b}
	Sorbitol + Glycerol	WPI	-15.75^{fgh}	-12.03^{kl}	0.0687^{fgh}
		WPI/MPC	-14.00^{c}	-10.34^{gh}	0.0645^{j}
63	Sorbitol	WPI	-14.09^{c}	-9.93^{ef}	0.0608^{kl}
		WPI/MPC	-13.01^{ab}	-9.82^{de}	0.0912^{c}
	Sorbitol + Glycerol	WPI	-15.00^{de}	-11.77^{k}	0.0661^{ghij}
		WPI/MPC	-15.50^{efg}	-10.63^{hi}	0.0603^{lm}
84	Sorbitol	WPI	-14.00^{c}	-8.21^{b}	0.0577^{m}
		WPI/MPC	-12.90^{a}	-7.42^{a}	0.0879^{d}
	Sorbitol + Glycerol	WPI	-14.70^{d}	-9.51^{cd}	0.0611^{kl}
		WPI/MPC	-15.01^{de}	-10.66^{hi}	0.0634^{jk}

$^{abc\ldots klm}$means within columns with same letter were not significantly different, $\alpha = 0.05$.

accounted for 0.38%, 0.58%, 0.58% and 0.49% of the total water in those bars, respectively.

During storage there was a slight decrease in quantity of intermediate water in all the bars (Figure 7.3). After 84 d storage, the level of intermediate water was Bar B >> Bar C = Bar D > Bar A. It would appear that after the first 21 d of storage, the effect of adding glycerol on increasing the intermediate water had diminished except when it was combined with use of the mixture of WPI and MPC as the protein source.

Bulk Water. High-protein nutrition bars such as those manufactured in this study, have <0.10% of the water that can be considered bulk water. On a solids basis this is only 0.01 gram of water per 100 gram of solids, or less (Table 7.10). Initially (i.e., d 1)

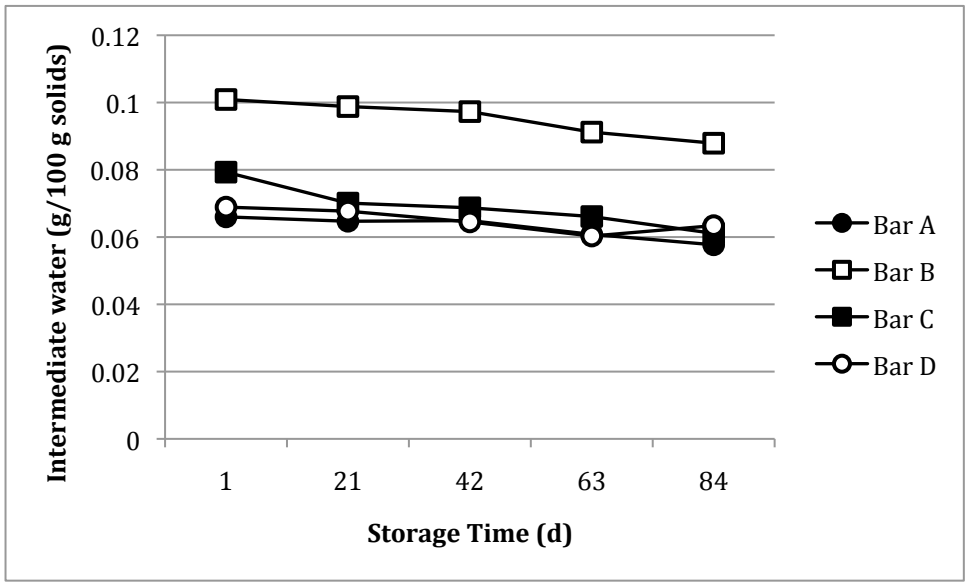

Figure 7.3 Change in intermediate water during 84 d at 35°C for (A) control high-protein nutrition bar containing whey protein isolate (WPI), sorbitol syrup and shortening compared to bars with (B) 50% of WPI substituted with milk protein concentrate (MPC), (C) 20% of sorbitol syrup replaced with glycerol, and (D) with both the MPC and glycerol substitutions, see Table 7.9 for significant differences.

Table 7.10 Onset and peak temperature for melting of bulk water and calculated quantity of bulk and bound water in high-protein nutrition bars of high-protein nutrition bars made using whey protein isolate (WPI) or a 50:50 WPI/milk protein concentrate (MPC) mixture, combined with shortening, and sorbitol syrup and glycerol (as described in Table 3.2) during 84 day of storage at 35°C.

Storage time (d)	Bar		Bulk Water			Bound Water (g/100 g solids)
			Onset Temp (°C)	Peak Temp (°C)	(g/100 g solids)	
1	Sorbitol	WPI	1.25^{efg}	4.16^{cd}	0.0104^{hij}	17.4465^{de}
		WPI/MPC	-0.29^{l}	2.09^{i}	0.0103^{hij}	17.5498^{b}
	Sorbitol + Glycerol	WPI	1.41^{def}	4.84^{a}	0.0096^{jk}	13.8971^{j}
		WPI/MPC	3.19^{a}	4.03^{d}	0.0084^{l}	14.0387^{g}
21	Sorbitol	WPI	0.92^{hi}	1.53^{k}	0.0123^{cde}	17.4460^{de}
		WPI/MPC	1.55^{cd}	4.46^{b}	0.0111^{fgh}	17.5511^{b}
	Sorbitol + Glycerol	WPI	1.47^{de}	3.76^{e}	0.0096^{jk}	13.9063^{i}
		WPI/MPC	1.02^{gh}	2.97^{gh}	0.0090^{kl}	14.0393^{g}
42	Sorbitol	WPI	0.66^{jk}	3.00^{gh}	0.0135^{ab}	17.4446^{e}
		WPI/MPC	-2.5^{n}	3.03^{g}	0.0120^{def}	17.5517^{b}
	Sorbitol + Glycerol	WPI	1.71^{bc}	4.38^{bc}	0.0108^{ghi}	13.9061^{i}
		WPI/MPC	-1.36^{m}	1.77^{j}	0.0101^{ij}	14.0414^{g}
63	Sorbitol	WPI	1.64^{cd}	3.97^{de}	0.0131^{bc}	17.4491^{cd}
		WPI/MPC	1.43^{def}	3.01^{g}	0.0115^{efg}	17.5583^{a}
	Sorbitol + Glycerol	WPI	0.77^{ij}	2.29^{i}	0.0116^{efg}	13.9083^{i}
		WPI/MPC	1.22^{fg}	2.88^{gh}	0.0097^{jk}	14.0460^{f}
84	Sorbitol	WPI	0.47^{k}	4.73^{a}	0.0142^{a}	17.4511^{c}
		WPI/MPC	1.20^{fg}	2.78^{h}	0.0127^{bcd}	17.5604^{a}
	Sorbitol + Glycerol	WPI	0.64^{jk}	2.22^{i}	0.0133^{b}	13.9116^{h}
		WPI/MPC	1.90^{b}	3.50^{f}	0.0109^{ghi}	14.0417^{g}

$^{abc\ldots ijk}$means within columns with same letter were not significantly different, $\alpha = 0.05$.

substituting MPC for 50% of the WPI had no significant effect on the level of bulk water. However, after the powders had become more solvated during the first 21 d of storage, there was a slight increase ($P < 0.05$) in bulk water for Bar A and Bar B, but there was less change in Bar B. For the remainder of storage, Bar B had slightly lower bulk water levels than Bar A. Adding glycerol lowered the amount of bulk water in the bars. Bar D containing MPC and glycerol, had significantly lower bulk water levels than the other bars. Although on a percentage basis, it was similar. Adding glycerol decreased the total amount of water in the bars, and initially Bars A, B, C and D contained only 0.0594%, 0.0583%, 0.0686% and 0.0595%, respectively, of their water as bulk water. The overall trend was for bulk water to increase during storage. On a solids basis, Bar A had the most and Bar D the least bulk water after 84 d (Figure 7-4). On a percentage of

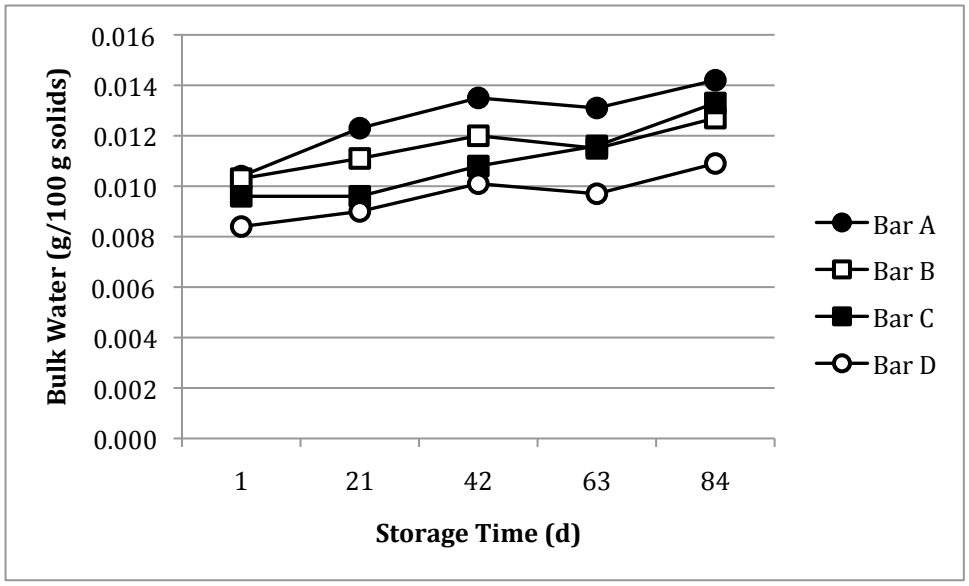

Figure 7.4 Change in bulk water during 84 d at 35°C for (A) control high-protein nutrition bar containing whey protein isolate (WPI), sorbitol syrup and shortening compared to bars with (B) 50% of WPI substituted with milk protein concentrate (MPC), (C) 20% of sorbitol syrup replaced with glycerol, and (D) with both the MPC and glycerol substitutions, see Table 7.9 for significant differences.

total water, adding MPC decreased the amount of bulk water while adding glycerol increased the amount of bulk water. At d 84, Bars A, B, C and D contained 0.0810%, 0.0719%, 0.0951% and 0.0772% of their water as bulk water, respectively.

Bound water. Total water content of the bars was calculated based upon the water content of the bar ingredients. Bound water was then calculated by difference from the bulk and intermediate water contents that were calculated from DSC thermograms. In each of the bars, bound water accounted for >99% of total water in the bars. The amount of bound water on a solids basis varied depending on bar formulation, and at d 1 was 17.447, 17.550, 13.897 and 14.039 g/100 g solids for Bars A, B, C and D, respectively. During storage there was an increase in amount of bound water for each of the bars by ~0.01 g/100 g of solids (Table 7.10). This was concomitant with a similar decrease in intermediate water (Table 7.9 and Figure 7.3), and an ~0.002 g/100 g solids increase in bulk water. Therefore, during storage there was an increase in interactions between water and the other components of the bars, such that some of the intermediate water molecules became strongly bound and were no longer freezable at -40°C.

State of Proteins

To gain information on how including glycerol as part of HPN bar formulations may influence stability of the proteins, and possible changes in proteins during storage, samples of bars were examined by DSC as shown in Figure 7.2. Two endothermic transitions, one with a peak temperature of ~65°C was assigned to unfolding of α-La, and the second with a peak temperature of ~76°C was assigned to unfolding of β-Lg. Adding either MPC and glycerol to the HPN bars caused significant changes to the onset and peak temperatures for both α-La ($P < 0.05$) and β-Lg ($P < 0.0001$) denaturation events

(Table 7.11), and the extent of their endothermic transitions (Table 7.12). There was also significant carbohydrate x protein interaction for α-La onset and peak temperatures, and for β-Lg onset temperature but not the peak temperature.

α-Lactalbumin. For the control HPN bar (made using WPI, sorbitol syrup and shortening), the onset for denaturation of α-La on d 1 after manufacture occurred at 62.0°C, with the peak occurring at 64.2°C (Table 7.13). Including MPC in the bar formulation (Bar B) caused unfolding of α-La to commence 2°C lower (P <0.05), but did not change the peak temperature. In contrast, adding glycerol (Bar C) had a significant but little effect on onset temperature, but increased the peak temperature to 65.9°C. The enthalpy changes for α-La denaturation were similar for all the bars at d 1, and for Bars A, B, C and D were 0.25, 0.23, 0.25 and 0.26 J/g protein, respectively.

During storage of the bars there was an increase (P < 0.001) during the first 42 d in both onset and peak temperature for α-La denaturation, and then it remained relatively constant. After 84 d, the onset temperatures were in the range of 64 to 66°C with Bar A > Bar B > Bar C > Bar D (Table 7.13). Peak temperatures after 84 d were in the range of 67 to 69°C with Bar B = Bar D > Bar A > Bar C. At the same time as the temperature at which unfolding occurred increased, there was decrease in the extent of unfolding as measured by enthalpy change. This occurred gradually during storage and by d 84, had dropped to 0.12, 0.18, 0.13 and 0.17 J/g protein for Bars A, B, C and D, respectively (Table 7.13). The largest change occurred in the two bars made using WPI only (Bars A and C), in which ΔH decreased by ~50%. There was no significant effect (P = 0.213) by addition of glycerol on the extent of enthalpic change during denaturation of α-La.

Table 7.11 ANOVA of dependent variable of onset and peak temperatures of thermal denaturation transitions for α-lactalbumin (α-La) and β-lactoglobulin (β-Lg) in high-protein nutrition bars made using whey protein isolate and milk protein concentrate with different combinations of sorbitol syrup and glycerol during 84 d storage at 35°C.

		P			
Effect	**df**	**α-La Onset (°C)**	**α-La Peak (°C)**	**β-Lg Onset (°C)**	**β-Lg Peak (°C)**
Carbohydrate (Carb)	1	0.0024	0.0065	<0.0001	<0.0001
Protein (Prot)	1	0.0148	0.0431	<0.0001	<0.0001
Carb x Prot	1	0.0010	0.0026	0.0008	0.3929
Time (T)	4	<0.0001	<0.0001	<0.0001	<0.0001
Carb x T	4	0.0282	0.0012	0.0004	0.9254
Prot x T	4	<0.0001	0.0060	<0.0001	0.0946
Carb x Prot x T	4	<0.0001	<0.0001	0.0063	0.1766

Table 7.12 ANOVA of dependent variable of denaturation enthalpy (ΔH) for α-lactalbumin (α-La) and β-lactoglobulin (β-Lg) in high-protein nutrition bars made using whey protein isolate and milk protein concentrate with different combinations of sorbitol syrup and glycerol during 84 d storage at 35°C.

		P	
Effect	**df**	**α-La ΔH (J/g protein)**	**β-Lg ΔH (J/g protein)**
Carbohydrate (Carb)	1	0.2130	<0.0001
Protein (Prot)	1	<0.0001	<0.0001
Carb x Prot	1	0.2978	<0.0001
Time (T)	4	<0.0001	<0.0001
Carb x T	4	0.0169	0.0728
Prot x T	4	<0.0001	0.0002
Carb x Prot x T	4	0.0037	0.1026

Table 7.13 Onset and peak temperatures (Temp), and enthalpic change (ΔH) for thermal denaturation of α-lactalbumin in high-protein nutrition bars made using whey protein isolate (WPI) or a 50:50 WPI/milk protein concentrate (MPC) mixture, combined with shortening, and sorbitol syrup with or without added glycerol (as described in Table 3.2) during 84 d of storage at 35°C.

Storage Time (d)	Bar		α-Lactalbumin Denaturation		
			Onset Temp (°C)	Peak Temp (°C)	ΔH (J/g protein)
1	Sorbitol	WPI	62.00^{k}	64.24^{h}	0.25^{a}
		WPI/MPC	60.20^{l}	64.83^{gh}	0.23^{b}
	Sorbitol + Glycerol	WPI	62.15^{ij}	65.89^{f}	0.25^{a}
		WPI/MPC	60.41^{l}	64.57^{h}	0.26^{a}
21	Sorbitol	WPI	63.93^{fgh}	67.14^{de}	0.22^{bc}
		WPI/MPC	62.19^{jk}	65.80^{fg}	0.21^{c}
	Sorbitol + Glycerol	WPI	63.00^{ij}	65.65^{fg}	0.21^{cd}
		WPI/MPC	61.99^{k}	66.16^{ef}	0.23^{b}
42	Sorbitol	WPI	66.65^{ab}	69.55^{a}	0.18^{fg}
		WPI/MPC	66.00^{bc}	68.78^{abc}	0.21^{cd}
	Sorbitol + Glycerol	WPI	63.33^{hi}	66.50^{ef}	0.17^{gh}
		WPI/MPC	67.10^{a}	68.85^{ab}	0.21^{cde}
63	Sorbitol	WPI	65.53^{dc}	68.94^{ab}	0.15^{i}
		WPI/MPC	63.61^{ghi}	68.26^{bc}	0.19^{ef}
	Sorbitol + Glycerol	WPI	63.0^{ij}	66.08^{f}	0.16^{hi}
		WPI/MPC	64.41^{efg}	68.86^{ab}	0.20^{de}
84	Sorbitol	WPI	65.94^{bc}	69.02^{ab}	0.12^{j}
		WPI/MPC	65.33^{cde}	69.40^{a}	0.18^{fg}
	Sorbitol + Glycerol	WPI	64.67^{def}	67.86^{cd}	0.13^{j}
		WPI/MPC	64.08^{fgh}	69.29^{a}	0.17^{gh}

$^{abc\ldots jkl}$means within columns with same letter were not significantly different, α = 0.05.

β-Lactoglobulin. The effects of bar formulation on thermal denaturation of β-Lg was similar to that for α-La, except that for β-Lg ,adding glycerol had a significant effect on ΔH (Table 7.11). There was not a significant carbohydrate x protein interaction with respect to the peak temperature for β-Lg.

Initially (d 1), there was no significant difference between the bars in the temperature at which β-Lg unfolding began, with onset temperature being 70°C. The peak temperatures were significantly different, with Bar B > Bar A = Bar D > Bar C, with values of 77.4, 76.2, 75.8 and 74.5°C, respectively (Table 7.14). During storage, the same change occurred as with α-La. Onset and peak temperatures gradually increased, with significant differences observed between the bars for onset temperature as well as peak temperature. After 84 d, these significant differences remained, and onset temperatures had increased from 3 to 6°C with Bar A, B, C and D having values of 73.4, 76.1, 71.0 and 74.7C, respectively. For peak temperature, there was a 2 to 3°C increase during storage, with the same differences that were present originally being present at the end of storage. By d 84, peak denaturation temperatures for β-Lg were 78.2, 80.8, 76.9 and 78.6°C for Bars A, B, C and D, respectively (Table 7.14).

Enthalpy related to β-Lg unfolding had similar relationship between the bar formulations as observed for α-La. The bars containing MPC (Bars B and D) had lower enthalpy than the bars made using WPI as the protein source (Table 7.14). A decrease in enthalpy was expected as MPC contains about 80% casein and only 20% whey proteins. When taking into account the presence of glycomacropeptide in whey, the β-Lg content of Bars B and D that use a 1:1 mixture of WPI and MPC, would be reduced by ~40% compared to Bars A and C.

Table 7.14 Onset and peak temperatures (Temp), and enthalpic change (ΔH) for thermal denaturation of β-Lactoglobulin in high-protein nutrition bars made using whey protein isolate (WPI) or a 50:50 WPI/milk protein concentrate (MPC) mixture, combined with shortening, and sorbitol syrup with or without added glycerol (as described in Table 3.2) during 84 d of storage at 35°C.

Storage Time (d)	Bar		β-Lactoglobulin Denaturation		
			Onset Temp (°C)	Peak Temp (°C)	ΔH (J/g protein)
1	Sorbitol	WPI	70.30^{gh}	76.23^{ghi}	1.01^{a}
		WPI/MPC	70.29^{gh}	77.42^{def}	0.75^{g}
	Sorbitol + Glycerol	WPI	69.56^{hi}	74.50^{k}	1.03^{a}
		WPI/MPC	70.32^{gh}	75.81^{hij}	0.59^{j}
21	Sorbitol	WPI	71.64^{f}	76.32^{ghi}	0.96^{b}
		WPI/MPC	73.72^{de}	78.50^{c}	0.71^{h}
	Sorbitol + Glycerol	WPI	69.20^{i}	75.06^{jk}	0.92^{b}
		WPI/MPC	73.32^{e}	76.95^{efg}	0.55^{k}
42	Sorbitol	WPI	73.27^{e}	78.22^{cd}	0.87^{c}
		WPI/MPC	75.16^{bc}	79.85^{ab}	0.65^{i}
	Sorbitol + Glycerol	WPI	69.70^{hi}	75.60^{ij}	0.85^{cd}
		WPI/MPC	75.43^{ab}	78.77^{c}	0.50^{l}
63	Sorbitol	WPI	73.24^{e}	77.99^{cde}	0.82^{de}
		WPI/MPC	75.59^{ab}	80.18^{a}	0.60^{j}
	Sorbitol + Glycerol	WPI	69.25^{i}	75.86^{hij}	0.81^{e}
		WPI/MPC	74.39^{cd}	78.98^{bc}	0.39^{m}
84	Sorbitol	WPI	73.35^{e}	78.23^{cd}	0.80^{ef}
		WPI/MPC	76.13^{a}	80.82^{a}	0.62^{j}
	Sorbitol + Glycerol	WPI	71.00^{fg}	76.85^{fgh}	0.77^{fg}
		WPI/MPC	74.69^{bc}	78.64^{c}	0.41^{m}

$^{abc\ldots jklm}$means within columns with same letter were not significantly different, α = 0.05.

At d 1 after manufacture, the β-Lg denaturation enthalpy was 1.0 J/g protein for Bars A and C, while for Bar B it was 0.75 J/g protein, and Bar D was even lower at 0.59 J/g protein. When using only WPI, adding glycerol had no impact on enthalpy, but it lowered the enthalpy when MPC was added as part of the bar formulation (Table 7.14). During storage of the bars, the denaturation enthalpy for β-Lg decreased by about 20% for all the bars, in a similar manner as for α-La (see Appendix B, Figures B.1 and B.2).

Disulfide Bonding

Sulfhydryl Groups. Including MPC in HPN bar formulations significantly ($P \leq 0.01$) affected the quantity of free sulfhydryl, total cysteine and cystine amino acids sulfhydryl groups, and disulfide bonds (Table 7.15). Adding glycerol did not change the total number of sulfhydryl groups as this is a function of protein content and not carbohydrate content. Glycerol did have a significant effect on quantity of free sulfhydryl groups ($P = 0.019$) (Table 7.15), and had a tendency to change the calculated number of disulfide bonds ($P = 0.062$). There was also a significant carbohydrate x

Table 7.15 ANOVA of dependent variables for high-protein nutrition bar free sulfhydryl group, total sulfhydryl group, and disulfide bond values.

Effect	*P*			
	df	Free SH	Total SH	S-S
Carbohydrate (Carb)	1	0.0188	0.6732	0.0622
Protein (Prot)	1	0.0003	0.0135	0.0130
Carb x Prot	1	0.0913	0.6007	0.3508
Time (T)	4	0.9022	0.8981	0.6125
Carb x T	4	0.9909	0.9125	0.9790
Prot x T	4	0.9991	0.9963	0.9716
Carb x Prot x T	4	0.9941	0.9481	0.9771

protein interaction affect on free sulfhydryl groups, but not on total sulfhydryl groups of on disulfide bonds.

Even though there were less whey proteins in MPC than WPI, and therefore an expected lower amount of sulfhydryl groups, there was no significant difference (at $\alpha = 0.05$) in total sulfhydryl groups between any of the bar samples (Table 7.16). Although, the bars containing MPC were consistently numerically lower by 3 to 5%. It was expected that since the sulfhydryl groups occur more in the whey proteins than in the caseins, and MPC has only ~20% of its proteins as whey proteins, that there would be a significant difference. Maybe there were some confounding factors related to the different components of the bars, such as the extent of brown pigments produced during storage that have absorbance at 412 nm (the wavelength at which NTB^{2-} concentration was measured). During storage, time had no effect on number of free sulfhydryl groups ($P = 0.9$), total sulfhydryl groups ($P = 0.9$) or disulfide bonds ($P = 0.6$). Neither were there any significant interactions with time and bar components.

SDS-PAGE. Standard SDS-PAGE, as well as SDS-PAGE with DTT included to reduce and break disulfide bonds, was used to exam possible disulfide bond formation and its effect on the structural changes (hardening) of HPN bars during storage. As well as a molecular weight marker, WPI and MPC powders were included on the gels as a reference. Based on Loveday and others (2009, 2010) presumptive identification was given to six bands as bovine serum albumin (BSA), α-casein, β-casein, κ-casein, β-Lg and α-La (Figures 7.5 and 7.6). Overall there were no differences between the bands in the gels for Bar A and Bar C (made using WPI), or Bar B and Bar D (made using WPI

Table 7.16 Concentration of free sulfhydryl (Free SH), total sulfhydryl (Total SH), and disulfide (S-S) bonds of high-protein nutrition bars made using whey protein isolate (WPI) or a 50:50 WPI/milk protein concentrate (MPC) mixture combined with shortening, sorbitol syrup, and glycerol (as described in Table 3.2) during 84 day of storage at 35°C.

Storage Time (d)	Bar		Sulfhydryl Groups		
			Free SH µmoles/g protein	Total SH µmoles/g protein	S-S µmoles/g protein
1	Sorbitol	WPI	1397.0^{abc}	2196.2^{a}	400.5^{d}
		WPI/MPC	1164.2^{de}	2136.5^{a}	486.1^{abc}
	Sorbitol + Glycerol	WPI	1448.6^{a}	2257.3^{a}	396.1^{d}
		WPI/MPC	1304.4^{abcd}	2181.4^{a}	439.4^{abcd}
21	Sorbitol	WPI	1383.1^{abc}	2230.1^{a}	423.8^{bcd}
		WPI/MPC	1174.6^{de}	2143.4^{a}	484.4^{abc}
	Sorbitol + Glycerol	WPI	1432.5^{ab}	2220.7^{a}	394.4^{d}
		WPI/MPC	1287.1^{bcde}	2134.1^{a}	423.8^{bcd}
42	Sorbitol	WPI	1394.7^{abc}	2224.6^{a}	414.9^{cd}
		WPI/MPC	1153.3^{de}	2139.3^{a}	493.6^{ab}
	Sorbitol + Glycerol	WPI	1425.3^{ab}	2230.9^{a}	403.3^{d}
		WPI/MPC	1279.5^{bcde}	2142.8^{a}	431.9^{abcd}
63	Sorbitol	WPI	1384.7^{abc}	2237.9^{a}	426.6^{bcd}
		WPI/MPC	1142.3^{e}	2131.3^{a}	494.5^{ab}
	Sorbitol + Glycerol	WPI	1374.7^{abc}	2191.2^{a}	396.1^{d}
		WPI/MPC	1264.6^{cde}	2160.7^{a}	448.0^{abcd}
84	Sorbitol	WPI	1391.4^{abc}	2269.0^{a}	439.4^{abcd}
		WPI/MPC	1148.4^{de}	2145.3^{a}	498.8^{a}
	Sorbitol + Glycerol	WPI	1413.1^{abc}	2245.1^{a}	415.7^{cd}
		WPI/MPC	1278.4^{bcde}	2190.6^{a}	456.1^{abcd}

^{abcde}Means with the same letter within columns were not significantly different, $\alpha = 0.05$

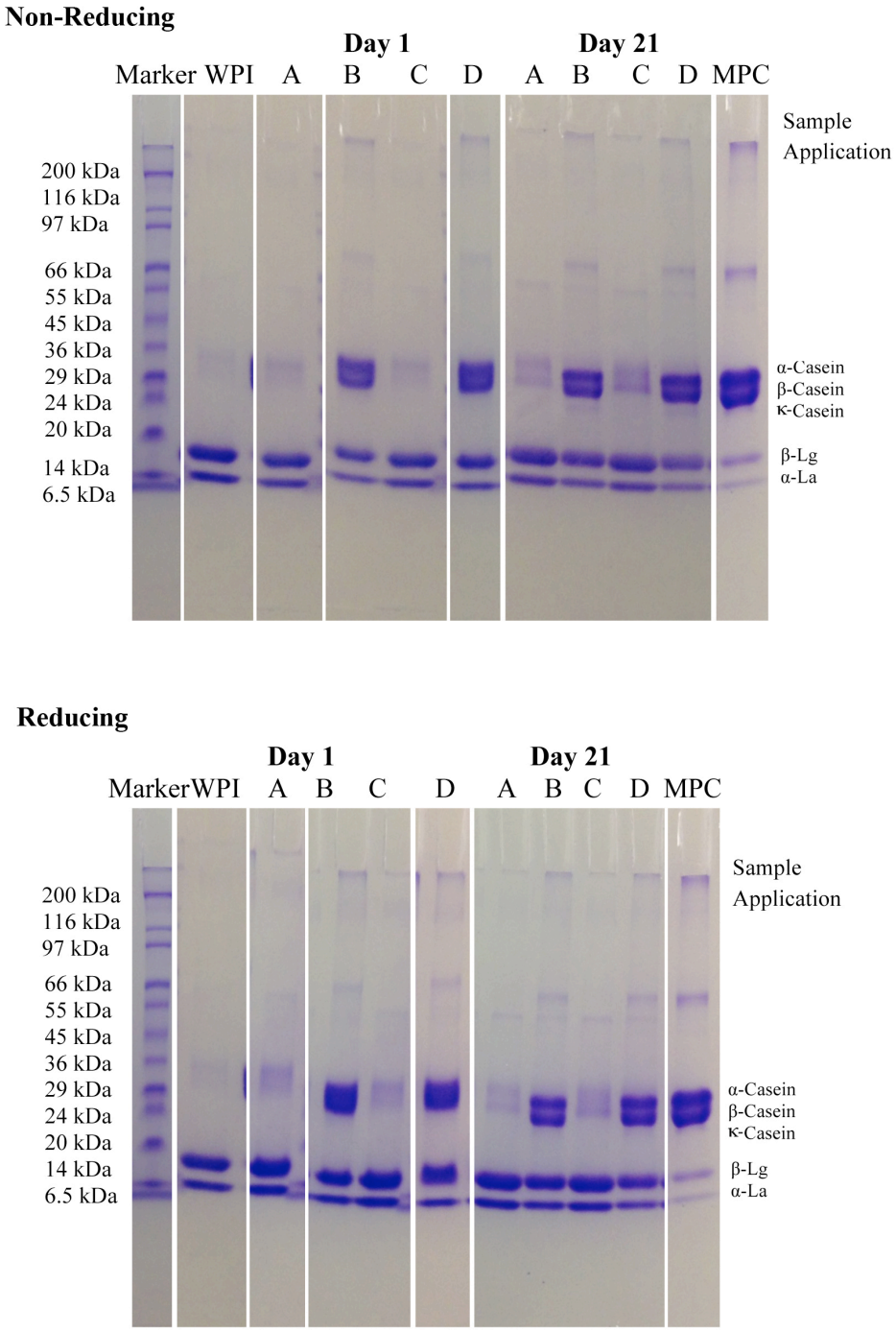

Figure 7.5 SDS-PAGE using non-reducing and reducing conditions of (A) a control high-protein nutrition bar (38% whey protein isolate powder (WPI)) , 44% sorbitol syrup, 18% vegetable shortening) compared to bars in which (B) 50% of WPI was replaced with milk protein concentrate (MPC), (C) 20% of sorbitol syrup was replaced with glycerol, and (D) that had both MPC and glycerol substitutions, at 1 and 21 d after manufacture and stored at 35°C.

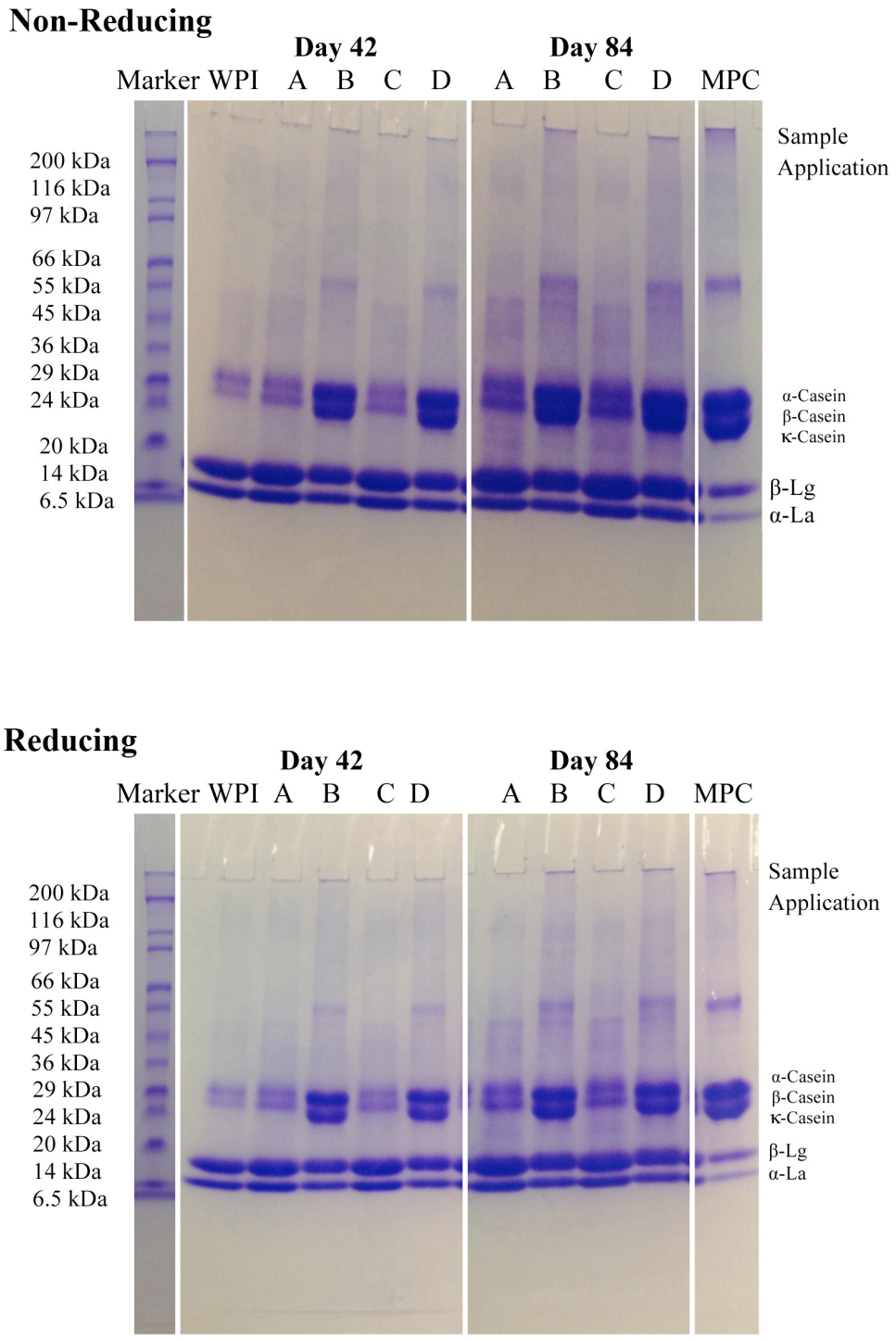

Figure 7.6 SDS-PAGE using non-reducing and reducing conditions of (A) a control high-protein nutrition bar (38% whey protein isolate powder (WPI)) , 44% sorbitol syrup, 18% vegetable shortening) compared to bars in which (B) 50% of WPI was replaced with milk protein concentrate (MPC), (C) 20% of sorbitol syrup was replaced with glycerol, and (D) that had both MPC and glycerol substitutions, after 42 and 84 d storage at 35° C.

and MPC), even though there were differences in the hardness of the bars. Nor were there any differences between the bands based on using non-reducing or reducing conditions. At d 84, there was more streaking but this was probably an artifact, as the MPC standard had the same smearing.

To confirm the lack of aggregation of whey proteins via inter-molecular disulfide bonding, SDS-PAGE was performed on Bar 1 and Bar 2 samples from Chapter 5 that had been frozen after 0, 15, 60 and 90 d of storage at 35°C. There was no difference in the bands observed between the samples that were reduced using DTT, or those that were not (Figure B.9). This confirms that disulfide bond formation between proteins during storage, was not the cause of bar hardening. This agrees with Loveday and others (2010).

Solubility in Urea and DTT. To further test for the formation of inter-molecular disulfide bonds in HPN bars that could be related to hardened during storage, some of the bars that had been kept in storage (~2 years) from Chapter 5 were tested for their solubility or insolubility in water, in water containing DTT, in 8 M urea, and in 8 M urea plus DTT. All of the bars responded in the same manner, including Bar 1 (Chapter 5) that was similar to Bar A, and Bar 3 (Chapter 5) that was similar to Bar C. Adding DTT to reduce disulfide bonds had no impact on protein solubility. The protein in the bars was only partially soluble when dispersed in water or 5 mM DTT solutions, and a large pellet was observed after centrifugation (see Figure 7.7A and B). In contrast the protein in the bars was completely soluble in 8 M urea (see Figure 7.7C and D).

Microstructure

Microstructure of Bars A, B, C and D immediately after manufacture, and during the 84 d storage at 35°C, was similar to the structure previously observed for Bars

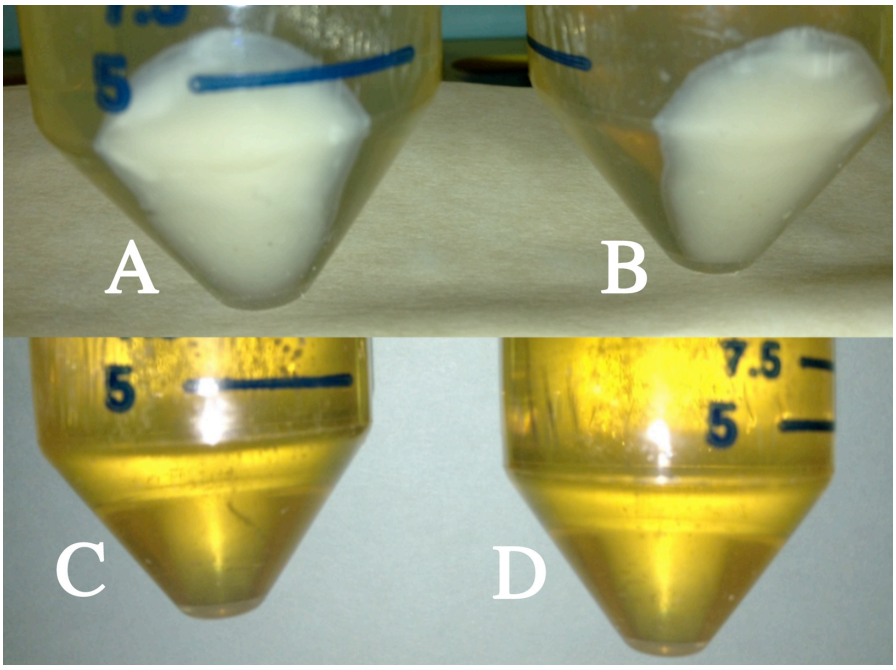

Figure 7.7 Presence or absence of insoluble pellet after centrifuging 2-year-old HPN bar that had been dispersed in (A) water, (B) 5 mM dithiothreitol, (C) 8 M urea and (D) 8 M urea plus 5 mM dithiothreitol.

1, 3 and 4 that were made using WPI and described in Chapter 5. Because the fluorescent stains that were used, Nile Red and FITC, fluoresce in the presence of lipids and protein, respectively, it was not possible to distinguish between fluorescence that was related to areas of the image that contained caseins, from areas of the image containing whey proteins such as β-Lg and α-La. Bar microstructure images obtained using CLSM are shown in Figure 7.8. Initially after manufacture, it was observed that there was a dispersion of the ingredients together after mixing. There were air voids (black) distributed throughout the bar and these typically were encased within the shortening lipid material (orange). In Bar B and D (made using the WPI/MPC mixture), the large

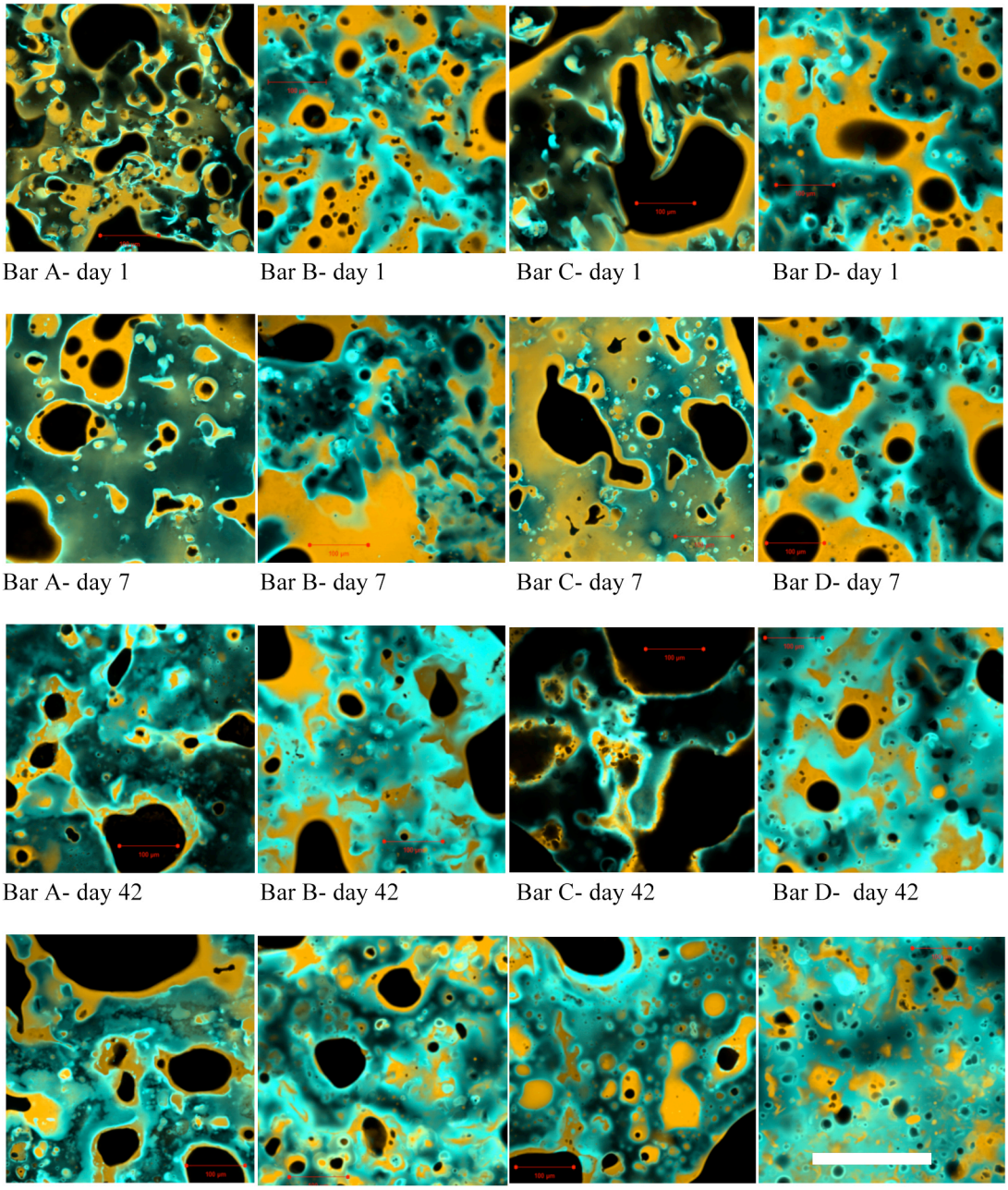

Figure 7.8 Confocal laser scanning micrographs of (Bar A) a control high-protein nutrition bar (38% whey protein isolate powder (WPI)) , 44% sorbitol syrup, 18% vegetable shortening) compared to bars in which (Bar B) 50% of whey protein isolate was replaced with milk protein concentrate (MPC), (Bar C) 20% of sorbitol syrup was replaced with glycerol, and (Bar D) that had both MPC and glycerol substitutions, during storage for 84 d at 35°C, false colorized with orange = lipid, cyan = protein, black = absence of fluorescence, bar = 200 µm

irregular shaped air voids were not as prevalent but there were numerous very small air voids contained within the same lipid mass

In Bar A and C that were made using WPI, the level of fluorescence from FITC (cyan) was lower, and the protein/solvent/cosolvent regions in the images tended to be darker. These bars were considerable harder than Bars B and D, with hardness values of ~400 compared to ~50 g-force. Perhaps the FITC solution was less able to penetrate into the hard protein network portion of the bars. Some extra time was allowed for stain penetration, which slightly improved fluorescence from the protein matrix ,but did not completely alleviate the problem. That this occurred on d 1 after manufacture, is an indication that this low intensity fluorescence from within the protein matrix was not caused by a phase separation that occurred during storage. Although there still may be portions of the sorbitol syrup, or sorbitol syrup/glycerol mixture, that are containing less protein powder than other portions.

When MPC and WPI were added together in the bar formulations (i.e., Bar B and D), there was no appearance of non-solubilized protein powder particles as observed in Bar 2 in Chapter 5. This is a limitation of the CLSM method in that FITC will bind to proteins in both WPI and MPC and fluoresce when excited. Consequently, there would be fluorescence from both any remaining protein powder particles as well the proteins that have been solvated and dispersed throughout the mass of solvent/cosolvent. Then when the fluorescence is captured, it appears as a continuous protein phase.

During the first week of storage, it appears that the components have become more solvated as observed in Chapter 5. Then with further storage, there was not a lot of changes in the CLSM images. In general, the protein/solvent/cosolvent matrix appeared

more extensive and continuous, and images for Bars A, B and C were all similar. Bar D, which had retained the most softness (i.e., it had less hardening during storage) had more extensive staining and fluorescence from FITC. This may be a consequence of easier penetration of the FITC into the softer textured bar sample.

DISCUSSION

WPI and MPC

As was shown in Chapter 6, mixing WPI and MPC in HPN bars can soften the bars and slow down hardening, and the optimum occurred when mixed together on a 1:1 ratio. It can be assumed from this that the MPC protein powder particles, and perhaps solvated casein micelles that become dispersed from the powder, act to interrupt formation of the extensive protein network structure formed by WPI proteins during bar storage. A microscopic method other than CLSM using fluorescence from FITC (or similar protein fluorophore) is needed to confirm this is happening. However, there is no reason why the MPC added into Bars B and D, would be dispersed any differently from what was observed in Bar 2 in Chapter 5.

Using a 1:1 mixture of WPI and MPC was very effective in lowering the initial hardness of the bars by ~6 times, and retarded hardness such that Bar B after 84 d storage at 35°C was still softer than Bar A at d 1 of storage. Going to higher proportions of MPC, such as 1:4 (WPI:MPC), had a negative impact (see Chapter 6) as the MPC becomes the predominant protein making up the bar matrix. Such bars are hard in structure, and take on the brittle crumbliness of bars made using 100% MPC as seen in Bar 2 (Chapter 5).

Sorbitol and Glycerol

When a small proportion of glycerol is substituted for part of the sorbitol syrup in HPN bar formulations, it changes from a solvent-cosolvent system to a solvent-cosolvent-cosolvent system. The relative size of the molecules are sorbitol > glycerol > water. The sorbitol molecules will still therefore be preferentially excluded from around the protein surfaces, and within that exclusion layer will be a combination of water and glycerol molecules. Glycerol will be present as it is at a similar concentration as the water molecules. Since adding glycerol had an inhibitory effect on bar hardening, it implies that glycerol molecules are interfering with, or masking, the sites on the protein surface that participate in protein-protein interactions leading to aggregation and formation of an extensive protein network within the bar matrix.

This is opposite to what happens when glycerol is used as the entire carbohydrate syrup and hardening is accelerated (see Chapter 5). Therefore, it can be presumed that the glycerol molecules are interacting with different moieties on the protein surface when present with water compared to being used alone (Chapter 5) or in combination with sorbitol syrup (Chapters 6 and 7). In Chapter 5 it was thought that glycerol was only interacting electrostatically with the protein. As shown by Vagenende and others (2009), when glycerol interacts electrostatically with protein surface moieties it is oriented in a perpendicular orientation. As such, it creates a larger exclusion zone (4 Å) than would be expected based on the 2.3 Å radius for its average center of mass for random orientation (Figure 7.9).

Therefore, in a water-glycerol system, water will accumulate in the exclusion zone as shown by McClements (2002). In contrast, when sorbitol is included as a

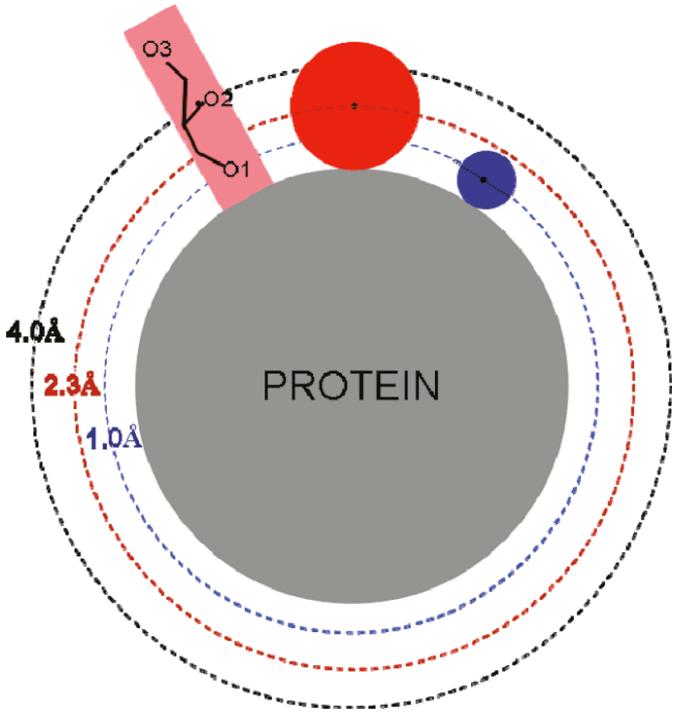

Figure 7.9 Schematic diagram showing exclusion zones around a protein surface of 1.0 Å for water molecules (blue dotted line) based on random center of mass (blue circle), 2.3 Å (red dotted line) based on center of mass for randomly oriented glycerol molecules, and 4.0 Å (black dotted line) for electrostatically interacting glycerol molecules oriented perpendicular (pink rectangle) to the protein surface, from Vagenende and others (2009).

cosolvent, the glycerol molecules can accumulate within the sorbitol exclusion zone, and given favorable circumstances can be oriented parallel to the protein surface. This occurs when the carbon atoms of glycerol can be oriented to lay across hydrophobic regions on the protein surface, as described by Vagenende and others (2009) (Figure 7.10). They proposed that glycerol molecules can act as an amphiphilic interface between the hydrophobic protein surface and the surrounding polar solvent/cosolvent. In this case, the three-carbon backbone of glycerol can lay across the protein surface, with the hydroxyl groups oriented outwards towards the water molecules. This would be a favorable orientation for glycerol in a system containing protein, water, glycerol and sorbitol as it

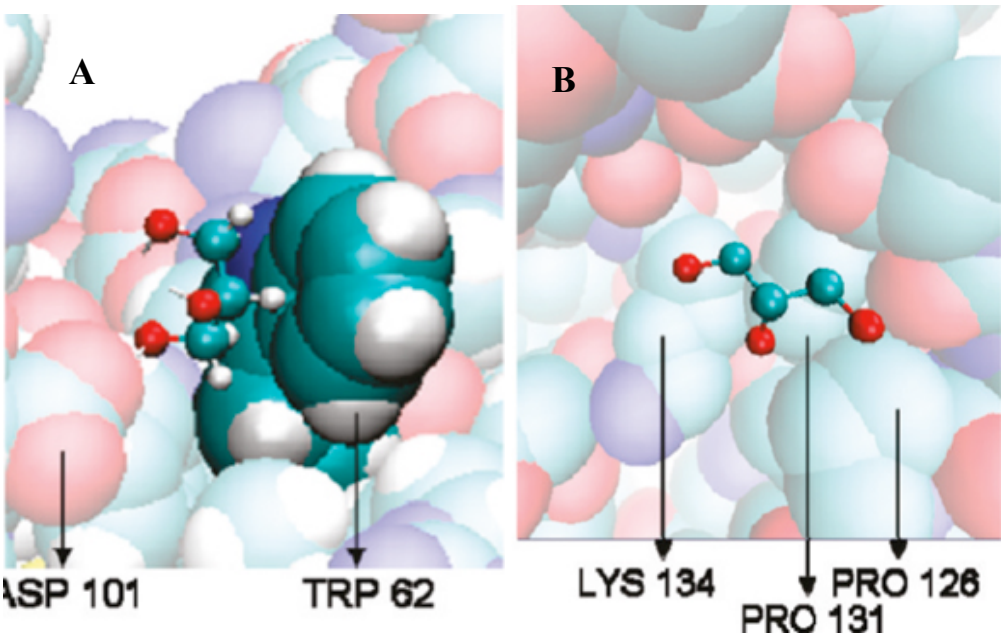

Figure 7.10 Modeling of a glycerol molecule with its hydrocarbon backbone in parallel orientation with the protein surface when placed (A) near a hydrophobic protein surface region containing tryptophan and with hydroxyl groups pointing away from the surface and towards and adjacent polar aspartate moiety, and (B) near hydrophobic surface region comprised of two prolines and the hydrocarbon side chain of a lysine side chain, as proposed by Vagenende and others (2009).

would release water molecules from being adjacent to hydrophobic moieties on the protein surface, and the entropic restrictions so induced.

Bar Hardening Mechanism

During storage of HPN bars, the temperature at which heat denaturation of the whey proteins (α-La and β-Lg) occurs increases slightly for all of the bar formulations. Concomitantly, there is a slight decrease in the change in denaturation enthalpy. This implies that the proteins move into a slightly more stable configuration during storage, and that less conformational changes take place. Since there are no intermolecular disulfide bonds being formed during storage, those conformational changes probably relate to having more stable configurations of the proteins in relation to the solvent/cosolvent mixture.

In Chapter 5, it was shown that phase separation is not the mechanism that promotes protein aggregation and HPN bar hardening. It is also apparent that bar hardening is not related to Maillard browning reactions (McMahon and others 2009). This leaves the entropic effects of hydrophobic interactions as being the driving force that promotes protein aggregation and causes bar hardening. The shielding of the hydrophobic regions on proteins by glycerol—when glycerol is at a lower proportion than sorbitol—diminishes the entropic effects of hydrophobic groups in relation to water. Therefore the proteins remain in a relatively non-aggregated form and bar hardening is reduced.

SUMMARY

It was confirmed that hardening of HPN bars can be retarded by replacing half of the WPI with MPC, and 20% of the sorbitol syrup with glycerol. The role of MPC was assumed to be related to the proteins from the MPC powder not completely dispersing into the sorbitol syrup/glycerol mixture. This would MPC powder particles still present within the protein matrix of the bars. This was not able to be discerned when MPC/WPI bars were examined using CLSM, but was presumed to be the case from observations on bars made using only MPC in Chapter 5.

No evidence of inter-molecular disulphide bond formation occurred during storage of HPN bars. Therefore, it was concluded that the protein aggregation that causes HPN bar hardening is driven by hydrophobic interactions between protein molecules.

It is hypothesized that adding glycerol with the sorbitol syrup retards bar hardening by masking hydrophobic regions on protein surfaces. This can only be achieved when glycerol is present along with water and a larger cosolvent such as sorbitol, and the glycerol molecules are oriented with their hydrocarbon backbone laying parallel to the hydrophobic moieties of the protein.

CHAPTER 8.

CONCLUSIONS

Based upon these studies, the following optimum components for HPN formulations were determined:

1. Combining WPI and MPC in a 1:1 ratio softens the bars compared to bars made using only WPI, while not imparting the crumbliness characteristic of bars made using MPC as the only source of protein. Bars made using the WPI/MPC mixture containing 38% (wt/wt) protein powder, had only 15% of the initial hardness of bars made using WPI. After 84 d storage at 35°C, the WPI/MPC bars were still softer than the WPI bars were at d 1.
2. Combining sorbitol syrup and glycerol in a 4:1 ratio reduces bar hardening during storage. Athough this slightly increased initial bar hardness, the bars after 84 d storage at 35°C were~30% less hard then their comparable bars made using only sorbitol syrup.

If glycerol was used as the only carbohydrate syrup source, the bars were initially very soft because of the low viscosity of glycerol, but the bars hardened very rapidly. Within a few days, such bars were harder than bars made using sorbitol syrup. When used as a combination with sorbitol syrup, the glycerol molecules apparently act differently with the proteins. Instead of promoting protein-protein interactions, they inhibit such interactions. There was not a major effect of glycerol on browning of the bars during storage, whether used at 100% or only 20% of the carbohydrate syrup component.

Overall, the extent of browning was minimal in all the bars tested in this research. This occurred because the only reducing sugars in the bars was the lactose contained in the protein powders. Even though MPC contains more lactose than WPI, there was less browning in the WPI/MPC bars during storage. This is probably because the MPC powder tended to remain in particulate form within the bars, without being completely dispersed or solvated by the solvent/cosolvent syrup. Thus, a portion of the lactose would have remained with those powder particles and not be mobile enough to react with amino groups on the proteins.

It was demonstrated that the mechanism of bar hardening is not a result of phase separation between the carbohydrate cosolvent and the protein. Rather, what had previously thought to be regions in the bars that contained the carbohydrate syrup and was devoid of protein, turned out to be an artifact and were air voids within the bar. These air voids (and hence black areas in CLSM images) were present in all bars, including initially after manufacture. The number and shape of the air voids seemed to be a function of the texture of the bars.

Aggregation of proteins via disulfide bonding (either formation of new disulfide linkages or through sulfhydryl interchange), was not related to bar hardening. No increase in number of disulfide bonds occurred during storage, whether the bars hardened extremely rapidly (as for bars made using glycerol only), or had very little hardening. Also, using DTT to reduce disulfide bonds had no impact on the solubility of bars that had hardened during storage, with much of the protein remaining insoluble. In contrast, all the protein in the bars was soluble when the bars were mixed with 8 M urea.

It is proposed that the hardening of HPN bar systems with water activity of 0.7 or less, involves aggregation of the proteins via hydrophobic interactions. In these high-protein-low water systems, almost all (>99.0%) of the water is accounted for as bound water that is strongly interacting with the cosolvent carbohydrate molecules. Less than 0.1% of the water molecules in HPN bars can be considered as bulk water, while ~0.6% can be classified as intermediate water, and is most likely to be those water molecules that are interacting with the protein molecules and are shielded from the cosolvent hydroxyl groups.

Interactions that occur on the surface of the proteins are the most critical for influencing bar hardening. Because of the high extent of electrostatic interactions that are possible with water and cosolvent molecules, most of the ionizable or dipole inducible moieties on the protein molecules will be oriented to participate in hydrogen bonding. As a consequence, hydrophobic moieties of the protein will cluster together to minimize decreases in entropy of any nearby water molecules. When sorbitol syrup is used as the carbohydrate, there would also be preferential exclusion of sorbitol molecules from near the protein surface (because of their larger size than water molecules) and most of the protein-solvent/cosolvent interactions would be with water molecules.

When glycerol is used as the carbohydrate syrup, the rate of hardening is accelerated. All of the electrostatic interactions with the proteins would be with the hydroxyl groups of glycerol and compared with water, glycerol has fewer possible interactions with other glycerol molecules and so there is a greater entropic effect with respect to hydrophobic moieties of the protein. When used in combination with sorbitol syrup, the smaller glycerol molecules are preferentially included around the protein

surface along with water molecules. In this situation, it is possible for glycerol to favorably interact with the protein hydrophobic moieties through its three-carbon backbone. At the same time, its hydroxyl groups extend away from the protein surface and are available for hydrogen bonding with surrounding sorbitol cosolvent molecules.

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APPENDICES

APPENDIX A. PHASE 1 EXPERIMENTS

Table A.1 ANOVA of dependent variables for high-protein nutrition bar bulk water values.

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	190	181.64	<0.0001
Temp	1	1	3.24	0.3227
Treatment*Temp	3	190	1.41	0.2408
Storage	7	190	29.83	<0.0001
Treatment*Storage	21	190	2.84	<0.0001
Temp*Storage	7	190	0.98	0.4459
Treatment*Temp*Storage	21	190	0.73	0.7958

Table A.2 ANOVA of dependent variables for high-protein nutrition bar intermediate water values.

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	190	3893.00	<0.0001
Temp	1	1	48.31	0.0910
Treatment*Temp	3	190	24.59	<0.0001
Storage	7	190	429.94	<0.0001
Treatment*Storage	21	190	25.87	<0.0001
Temp*Storage	7	190	9.76	<0.0001
Treatment*Temp*Storage	21	190	6.73	<0.0001

Table A.3 ANOVA of dependent variables for high-protein nutrition bar bound water values.

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	190	2.633E8	<0.0001
Temp	1	1	19.85	0.1405
Treatment*Temp	3	190	20.48	<0.0001
Storage	7	190	290.95	<0.0001
Treatment*Storage	21	190	22.57	<0.0001
Temp*Storage	7	190	7.38	<0.0001
Treatment*Temp*Storage	21	190	6.50	<0.0001

Table A.4 Change in intermediate water endotherm and quantity of intermediate water calculated as g/100g solids for control[1] high-protein nutrition bar compared to bars in which whey protein isolate was replaced with milk protein concentrate (MPC), sorbitol syrup was replaced with glycerol, or shortening was replaced with cocoa butter during 224 d storage at 22°C.

Storage Time (d)	Bar.	Onset 1 Temp °C	Peak 1 Temp °C	g. Intermediate water per 100 g Solids
0	1. Control	-19.30^{G}	-15.48^{G}	0.1742^{ab}
	2. MPC	-19.03^{EF}	-15.37^{EFG}	0.1674^{cdefg}
	3. Glycerol	-19.67^{H}	-15.90^{H}	0.1206^{yzA}
	4. Cocoa butter	-10.06^{d}	-5.51^{b}	0.1620^{hijklm}
6	1. Control	-15.67^{t}	-14.03^{uv}	0.1748^{ab}
	2. MPC	-14.60^{pq}	-13.53^{rst}	0.1670^{defgh}
	3. Glycerol	-17.91^{B}	-15.40^{FG}	0.1192^{yzA}
	4. Cocoa butter	-17.03^{x}	-15.11^{DEF}	0.1709^{abcde}
21	1. Control	-14.19^{n}	-12.60^{n}	0.1614^{ijklm}
	2. MPC	-14.49^{op}	-12.31^{mn}	0.1642^{ghijk}
	3. Glycerol	-13.40^{kl}	-10.70^{jk}	0.1172^{zA}
	4. Cocoa butter	-6.58^{a}	-5.00^{a}	0.1714^{abcd}
42	1. Control	-14.97^{r}	-13.55^{rst}	0.1629^{ghijk}
	2. MPC	-13.24^{jk}	-11.00^{kl}	0.1667^{defghi}
	3. Glycerol	-9.22^{c}	-4.95^{a}	0.1038^{B}
	4. Cocoa butter	-11.80^{h}	-9.20^{g}	0.1416^{rs}
70	1. Control	-14.69^{q}	-13.43^{pqrs}	0.1644^{fghijk}
	2. MPC	-12.91^{i}	-10.05^{i}	0.1489^{pq}
	3. Glycerol	-17.53^{zA}	-14.40^{xyzA}	0.0908^{C}
	Cocoa Butter	-17.53^{zA}	-14.40^{xyzA}	0.1396st
119	Control	-8.72^{b}	-7.04^{d}	0.1612^{jklm}
	WPI	-14.37^{o}	-13.11^{op}	0.1424^{rs}
	Glycerol	-15.15^{s}	-13.51^{qrs}	0.0836^{DE}
	Cocoa Butter	-13.53^{l}	-11.07^{l}	0.1265^{vwx}
175	Control	-16.35^{w}	-14.12^{uvxy}	0.1586^{lmn}
	WPI	-13.21^{j}	-11.12^{l}	0.1510^{pq}
	Glycerol	-10.89^{f}	-8.01^{e}	0.0835^{DE}
	Cocoa Butter	-13.10^{j}	-10.65^{j}	0.1243^{wxy}
224	Control	-19.40^{G}	-13.49^{qrs}	0.1532^{op}
	WPI	-17.99^{CB}	-12.10^{m}	0.1417^{rs}
	Glycerol	-16.17^{v}	-14.81^{BCD}	0.0785^{E}
	Cocoa Butter	-19.79^{HI}	-16.70^{I}	0.1260^{vwx}

[1]33.9% WPI, 46.7% sorbitol syrup, 19.4% vegetable shortening

$^{abc\ldots xyzABC}$ means within columns in Tables A.4 and A.5 with same letter were not significantly different, $\alpha = 0.05$

Table A.5 Change in intermediate water endothermic transition and quantity of intermediate water calculated as g/100g solids for a control[1] high-protein nutrition bar compared to bars in which whey protein isolate was replaced with milk protein concentrate (MPC), sorbitol syrup was replaced with glycerol, or shortening was replaced with cocoa butter during 90 d storage at 35°C.

Storage Time (d)	Bar.	Onset 1 Temp °C	Peak 1 Temp °C	g. Intermediate water per 100 g Solids
0	1. Control	-19.30^{G}	-15.48^{G}	0.1742^{ab}
	2. MPC	-19.03^{EF}	-15.37^{EFG}	0.1674^{cdefg}
	3. Glycerol	-19.67^{H}	-15.90^{H}	0.1206^{yzA}
	4. Cocoa butter	-10.06^{d}	-5.51^{b}	0.1620^{hijklm}
2	1. Control	-15.28^{s}	-14.29^{vwxy}	0.1754^{a}
	2. MPC	-17.31^{y}	-14.64^{zAB}	0.1660^{efghij}
	3. Glycerol	-17.91^{B}	-15.40^{FG}	0.1160^{A}
	4. Cocoa butter	-17.32^{y}	-15.00^{CD}	0.1677^{cdefg}
8	1. Control	-19.91^{IJ}	-14.71^{ABC}	0.1736^{ab}
	2. MPC	-17.70^{A}	-13.84^{tu}	0.1655^{defghij}
	3. Glycerol	-19.36^{G}	-14.07^{uvw}	0.1046^{B}
	4. Cocoa butter	-20.02^{J}	-16.71^{I}	0.1537^{nop}
15	1. Control	-20.04^{J}	-14.48^{yzA}	0.1727^{abc}
	2. MPC	-18.90^{DE}	-14.66^{zAB}	0.1510^{pq}
	3. Glycerol	-19.02^{EF}	-10.13^{i}	0.1004^{B}
	4. Cocoa butter	-15.98^{u}	-13.54^{rst}	0.1291^{vw}
29	1. Control	-14.01^{m}	-13.60st	0.1697^{bcdef}
	2. MPC	-19.09^{F}	-13.19^{opq}	0.1487^{pq}
	3. Glycerol	-14.76^{q}	-7.75^{e}	0.0842^{D}
	Cocoa Butter	-18.77^{D}	-12.56^{n}	0.1235^{xy}
43	Control	-14.97^{r}	-13.00^{o}	0.1594^{klm}
	WPI	-17.40^{yz}	-13.24^{opqr}	0.1537^{nop}
	Glycerol	-15.13^{rs}	-8.61^{f}	0.0862^{CD}
	Cocoa Butter	-16.98^{x}	-14.00^{uv}	0.1299^{uv}
60	Control	-11.90^{h}	-9.00^{g}	0.1572^{mno}
	WPI	-12.89^{i}	-6.00^{c}	0.1504^{pq}
	Glycerol	-11.56^{g}	-8.50^{f}	0.0690^{F}
	Cocoa Butter	-10.31^{e}	-8.00^{e}	0.1226^{xyz}
90	Control	-14.43^{op}	-13.03^{o}	0.1462^{qr}
	WPI	-18.12^{C}	-15.06^{DE}	0.1348^{tu}
	Glycerol	-16.36^{w}	-9.63^{h}	0.0717^{F}
	Cocoa Butter	-19.73^{HI}	-14.38^{wxyz}	0.1041^{B}

[1]33.9% WPI, 46.7% sorbitol syrup, 19.4% vegetable shortening

$^{abc…xyzABC}$ means within columns in Tables A.4 and A.5 with same letter were not significantly different, $\alpha = 0.05$

Table A.6 Change in bulk water endothermic transition and quantity of bulk water and bound calculated as g/100g solids for a control[1] high-protein nutrition bar compared to bars in which whey protein isolate was replaced with milk protein concentrate (MPC), sorbitol syrup was replaced with glycerol, or shortening was replaced with cocoa butter during 224 d storage at 22°C.

Storage Time (d)	Bar.	Onset 2 Temp °C	Peak 2 Temp °C	Bulk water g/100 g Solids	Bound water g/100 g Solids
0	1. Control	-2.14^{x}	0.35^{E}	0.0193^{lmnopqrs}	18.2194^{op}
	2. MPC	-2.47^{z}	-0.70^{j}	0.0185^{opqrstuv}	18.4661^{e}
	3. Glycerol	-2.41^{z}	1.44^{q}	0.0145^{zABCD}	1.4190^{v}
	4. Cocoa butter	-2.28^{y}	2.75^{g}	0.0182^{qrstuvwx}	18.2329^{kl}
6	1. Control	1.03^{a}	1.64^{o}	0.0185^{pqrstuvw}	18.2198^{op}
	2. MPC	0.47^{bc}	1.89^{n}	0.0179^{rstuvwx}	18.4671^{e}
	3. Glycerol	-1.73^{s}	0.22^{f}	0.0137^{BCD}	1.4211^{v}
	4. Cocoa butter	0.50^{b}	3.62^{e}	0.0152^{yzABCD}	18.2269^{mn}
21	1. Control	0.47^{bc}	1.30^{r}	0.0208^{ghijklmnop}	18.2307^{lm}
	2. MPC	-1.80^{t}	0.16^{G}	0.0190^{nopqrstuv}	18.4688^{e}
	3. Glycerol	-1.53^{q}	0.38^{DE}	0.0133^{D}	1.4235^{v}
	4. Cocoa butter	0.29^{d}	4.10^{c}	0.0192^{mnopqrst}	18.2225^{no}
42	1. Control	-0.13^{ij}	0.72^{w}	0.0203^{ijklmnopqr}	18.2299^{lm}
	2. MPC	-0.35^{l}	0.63^{x}	0.0192^{lmnopqrs}	18.4660^{e}
	3. Glycerol	-1.98^{v}	2.21^{l}	0.0140^{ABCD}	1.4361^{u}
	4. Cocoa butter	-0.15^{ijk}	0.49^{AB}	0.0182^{qrstuvwx}	18.2532^{t}
70	1. Control	-0.17^{jk}	0.73^{w}	0.0227^{abcdefgh}	18.2259^{mn}
	2. MPC	-2.46^{z}	-0.36^{i}	0.0204^{hijklmnopq}	18.4827^{dc}
	3. Glycerol	-0.14^{ijk}	1.50^{p}	0.0134^{CD}	1.4499^{t}
	Cocoa Butter	-0.33^{l}	0.86^{v}	0.0213^{efghijklmn}	18.2521^{t}
119	Control	-0.20^{k}	0.43^{cd}	0.0236^{abcdef}	18.2283^{lm}
	WPI	-0.06^{hg}	0.97^{tu}	0.0215^{efghijklm}	18.4881^{bc}
	Glycerol	-1.30^{o}	-0.09^{h}	0.0144^{zABCD}	1.4560^{rs}
	Cocoa Butter	-0.20^{k}	0.63^{x}	0.0219^{cdefghijk}	18.2647^{gh}
175	Control	-0.33^{l}	0.66^{x}	0.0242^{abcd}	18.2302^{lm}
	WPI	-0.02^{gf}	0.92^{u}	0.0213^{efghijklmn}	18.4797^{d}
	Glycerol	-2.26^{y}	1.16^{s}	0.0161^{wxyzA}	1.4544^{rst}
	Cocoa Butter	-0.54^{n}	5.27^{a}	0.0232^{abcdefg}	18.2656^{gh}
224	Control	-2.57^{A}	0.94^{u}	0.0227^{abcdefgh}	18.2371^{jk}
	WPI	-0.60^{n}	3.67^{e}	0.0210^{ghijklmno}	18.4893^{ab}
	Glycerol	-1.74st	0.15^{G}	0.0166^{uvwxyz}	1.4589^{r}
	Cocoa Butter	-2.28^{y}	2.12^{m}	0.0231^{abcdefg}	18.2639^{gh}

[1]33.9% WPI, 46.7% sorbitol syrup, 19.4% vegetable shortening

$^{abc\ldots xyzABC}$ means within columns in Tables A.6 and A.7 with same letter were not significantly different, $\alpha = 0.05$

Table A.7 Change in bulk water endothermic transition and quantity of bulk water and bound calculated as g/100g solids for a control[1] high-protein nutrition bar compared to bars in which whey protein isolate was replaced with milk protein concentrate (MPC), sorbitol syrup was replaced with glycerol, or shortening was replaced with cocoa butter during 90 d storage at 35°C.

Storage Time (d)	Bar.	Onset 2 Temp °C	Peak 2 Temp °C	Bulk water g/100 g Solids	Bound water g/100 g Solids
0	1. Control	-2.14^{x}	0.35^{E}	0.0193^{mnopqrs}	18.2195^{op}
	2. MPC	-2.47^{z}	-0.70^{J}	0.0185^{opqrstuvw}	18.4661^{e}
	3. Glycerol	-2.41^{z}	1.44^{q}	0.0145^{zABCD}	1.4190^{v}
	4. Cocoa butter	-2.28^{y}	2.75^{g}	0.0182^{qrstuvwx}	18.2329^{kl}
2	1. Control	-2.96^{B}	-1.19^{K}	0.0197^{klmnopqr}	18.2179^{op}
	2. MPC	0.26^{d}	1.89^{n}	0.0189^{nopqrstuv}	18.4672^{e}
	3. Glycerol	-1.75st	0.22^{F}	0.0159^{xyzAB}	1.4221^{v}
	4. Cocoa butter	0.41^{c}	3.63^{e}	0.0170^{stuvwxy}	18.2284^{lm}
8	1. Control	-1.44^{p}	2.47^{i}	0.0218^{defghijkl}	18.2177^{op}
	2. MPC	0.02^{f}	2.64^{h}	0.0181^{qrstuvwx}	18.4674^{e}
	3. Glycerol	-0.05^{gh}	3.94^{d}	0.0157^{xyzABCD}	1.4337^{u}
	4. Cocoa butter	-2.18^{x}	2.39^{j}	0.0199^{jklmnopqr}	18.2394^{j}
15	1. Control	-0.59^{n}	2.31^{k}	0.0240^{abcd}	18.2163^{p}
	2. MPC	-0.15^{ijk}	1.42^{q}	0.0188^{opqrstuv}	18.4822^{d}
	3. Glycerol	-1.60^{r}	0.36^{E}	0.0158^{xyzABC}	1.4377^{u}
	4. Cocoa butter	0.10^{e}	1.11^{s}	0.0191^{mnopqrstu}	18.2649^{gh}
29	1. Control	-0.09^{hi}	1.17^{s}	0.0221^{cdefghijk}	18.2212^{nop}
	2. MPC	-0.14^{ijk}	2.99^{f}	0.0223^{bcdefghij}	18.4810^{d}
	3. Glycerol	-2.01^{vw}	-0.12^{H}	0.0149^{yzABCD}	1.4550^{rst}
	Cocoa Butter	-1.34^{o}	4.45^{b}	0.0210^{ghijklmno}	18.2685^{g}
43	Control	-0.57^{n}	0.92^{u}	0.0226^{abcdefghi}	18.2310^{lm}
	WPI	-0.46^{m}	0.76^{w}	0.0205^{hijklmnopq}	18.4778^{d}
	Glycerol	0.29^{d}	1.14^{s}	0.0165^{vwxyzA}	1.4503st
	Cocoa Butter	-0.46^{m}	0.55^{az}	0.0215^{efghijklm}	18.2617^{h}
60	Control	-0.13^{ij}	0.41^{ED}	0.0250^{a}	18.2309^{lm}
	WPI	-0.13^{ij}	0.47^{BC}	0.0213^{fghijklmn}	18.4804^{d}
	Glycerol	-0.02^{fg}	0.63^{x}	0.0167^{tuvwxyz}	1.4682^{q}
	Cocoa Butter	-0.36^{l}	0.26^{F}	0.0245^{ab}	18.2659^{gh}
90	Control	-0.13^{ij}	0.64^{x}	0.0244^{abc}	18.2425^{j}
	WPI	-2.05^{w}	1.02^{t}	0.0223^{abcdefghi}	18.4950^{a}
	Glycerol	-1.87^{u}	0.61^{yx}	0.0172^{stuvwxy}	1.4651^{q}
	Cocoa Butter	-0.30^{l}	0.27^{yz}	0.0238^{abcde}	18.2851^{f}

[1]33.9% WPI, 46.7% sorbitol syrup, 19.4% vegetable shortening

$^{abc\ldots xyzABC}$ means within columns in Tables A.6 and A.7 with same letter were not significantly different, $\alpha = 0.05$

Table A.8 ANOVA of dependent variables for high-protein nutrition bar Intermediate water values.

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	190	3893.00	<0.0001
Temp	1	1	48.31	0.0910
Treatment*Temp	3	190	24.59	<0.0001
Storage	7	190	429.94	<0.0001
Treatment*Storage	21	190	25.87	<0.0001
Temp*Storage	7	190	9.76	<0.0001
Treatment*Temp*Storage	21	190	6.73	<0.0001

Table A.9 ANOVA of dependent variables for high-protein nutrition bar intermediate water enthalpic transition onset temperature values.

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	190	1286.06	<0.0001
Temp	1	1	14165.3	0.0053
Treatment*Temp	3	190	649.52	<0.0001
Storage	7	190	5206.96	<0.0001
Treatment*Storage	21	190	2505.52	<0.0001
Temp*Storage	7	190	5295.18	<0.0001
Treatment*Temp*Storage	21	190	974.04	<0.0001

Table A.10 ANOVA of dependent variables for high-protein nutrition bar intermediate water enthalpic transition peak temperature values.

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	190	694.96	<0.0001
Temperature (Temp)	1	1	290.80	0.0373
Treatment*Temp	3	190	336.57	<0.0001
Storage	7	190	1503.55	<0.0001
Treatment*Storage	21	190	829.56	<0.0001
Temp*Storage	7	190	1001.35	<0.0001
Treatment*Temp*Storage	21	190	401.28	<0.0001

Table A.11 ANOVA of dependent variables for high-protein nutrition bar bulk water values.

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	190	181.64	<0.0001
Temperature (Temp)	1	1	3.24	0.3227
Treatment*Temp	3	190	1.41	0.2408
Storage	7	190	29.83	<0.0001
Treatment*Storage	21	190	2.84	<0.0001
Temp*Storage	7	190	0.98	0.4459
Treatment*Temp*Storage	21	190	0.73	0.7958

Table A.12 ANOVA of dependent variables for high-protein nutrition bar bulk water enthalpic transition onset temperature.

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	190	3683.46	<0.0001
Temperature (Temp)	1	1	5.11	0.2652
Treatment*Temp	3	190	1579.97	<0.0001
Storage	7	190	7377.13	<0.0001
Treatment*Storage	21	190	908.85	<0.0001
Temp*Storage	7	190	1300.10	<0.0001
Treatment*Temp*Storage	21	190	2412.33	<0.0001

Table A.13 ANOVA of dependent variables for high-protein nutrition bar bulk water enthalpic transition peak temperature.

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	190	15043.6	<0.0001
Temperature (Temp)	1	1	18.43	0.1457
Treatment*Temp	3	190	1457.26	<0.0001
Storage	7	190	3664.33	<0.0001
Treatment*Storage	21	190	3321.38	<0.0001
Temp*Storage	7	190	5474.53	<0.0001
Treatment*Temp*Storage	21	190	3700.79	<0.0001

Table A.14 ANOVA of dependent variables for high-protein nutrition bar bound water values.

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	190	2.633E8	<0.0001
Temperature (Temp)	1	1	19.85	0.1405
Treatment*Temp	3	190	20.48	<0.0001
Storage	7	190	290.95	<0.0001
Treatment*Storage	21	190	22.57	<0.0001
Temp*Storage	7	190	7.38	<0.0001
Treatment*Temp*Storage	21	190	6.50	<0.0001

Table A.15 ANOVA of dependent variables for high-protein nutrition bar β-lactoglobulin denaturation enthalpy values.

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	190	3199.88	<0.0001
Temperature (Temp)	1	1	106.32	0.0615
Treatment*Temp	3	190	10.63	<0.0001
Storage	7	190	511.27	<0.0001
Treatment*Storage	21	190	35.06	<0.0001
Temp*Storage	7	190	14.51	<0.0001
Treatment*Temp*Storage	21	190	11.98	<0.0001

Table A.16 ANOVA of dependent variables for high-protein nutrition bar β-lactoglobulin denaturation onset temperature values.

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	189	18.38	<0.0001
Temperature (Temp)	1	1	31.00	0.1131
Treatment*Temp	3	189	25.38	<0.0001
Storage	7	189	19.14	<0.0001
Treatment*Storage	21	189	5.31	<0.0001
Temp*Storage	7	189	6.37	<0.0001
Treatment*Temp*Storage	21	189	12.20	<0.0001

Table A.17 ANOVA of dependent variables for high-protein nutrition bar β-lactoglobulin denaturation peak temperature values.

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	190	57.26	<0.0001
Temperature (Temp)	1	1	6.25	0.2423
Treatment*Temp	3	190	56.50	<0.0001
Storage	7	190	135.38	<0.0001
Treatment*Storage	21	190	17.40	<0.0001
Temp*Storage	7	190	10.72	<0.0001
Treatment*Temp*Storage	21	190	11.04	<0.0001

Table A.18 ANOVA of dependent variables for high-protein nutrition bar α -lactalbumin denaturation enthalpy values.

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	190	103.32	<0.0001
Temperature (Temp)	1	1	21.16	0.1363
Treatment*Temp	3	190	2.04	0.1100
Storage	7	190	95.63	<0.0001
Treatment*Storage	21	190	8.04	<0.0001
Temp*Storage	7	190	4.88	<0.0001
Treatment*Temp*Storage	21	190	2.52	0.0005

Table A.19 ANOVA of dependent variables for high-protein nutrition bar α -lactalbumin denaturation onset temperatures.

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	190	26.72	<0.0001
Temperature (Temp)	1	1	13.13	0.1714
Treatment*Temp	3	190	7.82	<0.0001
Storage	7	190	28.71	<0.0001
Treatment*Storage	21	190	6.71	<0.0001
Temp*Storage	7	190	1.22	0.2913
Treatment*Temp*Storage	21	190	6.67	<0.0001

Table A.20 ANOVA of dependent variables for high-protein nutrition bar α -lactalbumin denaturation peak temperatures.

Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	190	6.84	0.0002
Temperature (Temp)	1	1	26.38	0.1224
Treatment*Temp	3	190	20.39	<0.0001
Storage	7	190	53.25	<0.0001
Treatment*Storage	21	190	2.99	<0.0001
Temp*Storage	7	190	5.06	<0.0001
Treatment*Temp*Storage	21	190	7.62	<0.0001

SAS Statistics Code used for Analysis of Phase 1 Data:

```
%let resp = loghardness; /*change onset1 to the response
name to be analyzed  */
proc glimmix data = color plots = studentpanel; /*need to
specify the library of the data. */
     class treatment temp storage batch;
     model loghardness = treatment|temp|storage;
     random int temp /sub = batch;
     lsmeans treatment*temp*storage
/slicediff=storage*treatment adjust = tukey lines;
run;
title;
```

Table A.21 Change in denaturation enthalpy for α-lactalbumin of a control[1] high-protein compared to bars in which whey protein isolate was replaced with milk protein concentrate (MPC), sorbitol syrup was replaced with glycerol, or shortening was replaced with cocoa butter during 224 d storage at 22°C.

Time (d)	Bar	Onset Temp. (°C)	Peak Temp. (°C)	ΔH J/g protein
0	1. Control	61.47^{mnopqrstuv}	65.07^{vwxy}	0.24^{cdefg}
	2. MPC	62.03^{jklmnopqr}	66.00^{rstuvwx}	0.23^{ijklmnop}
	3. Glycerol	58.51^{zA}	65.76^{stuvwx}	0.25^{abc}
	4. Cocoa Butter	58.89^{yzA}	64.04^{y}	0.27^{ab}
6	1. Control	60.25^{rstuvwxyz}	65.94^{rstuvwx}	0.23^{ghijkl}
	2. MPC	63.63^{defghijk}	67.00^{klmnopqrst}	0.21^{nopqrstuv}
	3. Glycerol	59.13^{xyzA}	65.52^{tuvwxy}	0.27^{a}
	4. Cocoa Butter	59.16^{xyzA}	66.01^{rstuvwx}	0.24^{defghı}
21	1. Control	62.00^{jklmnopqr}	66.46^{opqrstuvwx}	0.25^{cdef}
	2. MPC	61.49^{mnopqrstuv}	67.17^{jklmnopqrs}	0.23$^{fghı jk}$
	3. Glycerol	59.36^{wxyzA}	67.00^{klmnopqrst}	0.25^{cde}
	4. Cocoa Butter	59.59^{vwxyzA}	65.83^{rstuvwx}	0.23^{jklmnopq}
42	1. Control	64.69^{bcdefg}	68.00^{ghijklmno}	0.23^{fghij}
	2. MPC	60.80^{pqrstuvwxy}	65.84^{rstuvwx}	0.21^{stuvwxy}
	3. Glycerol	60.87^{pqrstuvwx}	65.00^{xwy}	0.23^{ghijklm}
	4. Cocoa Butter	60.03^{stuvwxyz}	67.32^{ijklmnopqr}	0.23^{hijklmno}
70	1. Control	66.40^{ab}	70.00^{cd}	0.24^{efghı}
	2. MPC	60.41^{qrstuvwxyx}	65.76^{stuvwx}	0.22^{gklmnopqr}
	3. Glycerol	60.26^{rstuvwxyz}	65.50^{tuvwxy}	0.25^{bcd}
	4. Cocoa Butter	60.06^{tuvwxyz}	68.06^{fghijklmn}	0.21^{nopqrstuv}
119	1. Control	65.08^{abcde}	68.35^{efghijklm}	0.22^{jklmnopqrs}
	2. MPC	62.46^{ijklmnop}	66.63^{nopqrstuv}	0.21^{rstuvwxy}
	3. Glycerol	60.65^{pqrstuvwxy}	65.43^{uvwxy}	0.22^{lmnopqrstu}
	4. Cocoa Butter	61.00^{opqrstuvwx}	68.80^{defghı}	0.21^{opqrstuvw}
175	1. Control	66.83^{a}	68.62^{defghij}	0.21^{pqrstuvw}
	2. MPC	61.74^{klmnopqrst}	64.99^{wxy}	0.20^{xyzABC}
	3. Glycerol	62.07^{jklmnopqr}	64.00^{y}	0.24^{defgh}
	4. Cocoa Butter	61.50^{mnopqrstuv}	68.39^{efghijklm}	0.19^{ABC}
224	1. Control	60.89^{pqrstuvwx}	68.51^{defghijk}	0.21^{qrstuvw}
	2. MPC	61.62^{lmnopqrstu}	66.98^{klmnopqrst}	0.20^{yzABC}
	3. Glycerol	64.34^{cdefghi}	69.60^{def}	0.22^{jklmnopqrs}
	4. Cocoa Butter	65.53^{abcd}	71.46^{bc}	0.21^{tuvwxyz}

[1]33.9% WPI, 46.7% sorbitol syrup, 19.4% vegetable shortening.

$^{abc…xyzABC}$ means within columns in Tables A.21 and A.22 with same letter were not significantly different, α = 0.05

Table A.22 Change in denaturation enthalpy for β-lactoglobulin of a control[1] high-protein compared to bars in which whey protein isolate was replaced with milk protein concentrate (MPC), sorbitol syrup was replaced with glycerol, or shortening was replaced with cocoa butter during 90 d storage at 35°C.

Time (d)	Bar	Onset Temp. (°C)	Peak Temp. (°C)	ΔH J/g protein
0	1. Control	$80.77^{fghijklmnopq}$	82.82^{u}	0.89^{ab}
	2. MPC	78.31^{tuv}	87.48^{jklmn}	0.62^{tu}
	3. Glycerol	76.01^{xy}	80.40^{v}	0.75^{jklmn}
	4. Cocoa Butter	79.01^{qrstuv}	79.97^{v}	0.87^{bc}
2	1. Control	$81.17^{defghijklmn}$	83.43^{tu}	0.87^{bc}
	2. MPC	$80.00^{klmnopqrst}$	82.85^{u}	0.61^{uv}
	3. Glycerol	83.99^{a}	85.79^{rpq}	0.69^{pq}
	4. Cocoa Butter	78.71^{rstuv}	80.00^{v}	0.83^{de}
8	1. Control	$81.56^{cdefghijklm}$	84.82^{rst}	0.76^{hijkl}
	2. MPC	$81.77^{cdefghi}$	88.93^{efghig}	0.58^{wx}
	3. Glycerol	$81.59^{cdefghijklm}$	90.28^{abcde}	0.64^{st}
	4. Cocoa Butter	78.31^{tuv}	80.00^{v}	0.82^{ef}
15	1. Control	83.93^{a}	86.00^{nopqr}	0.77^{hijk}
	2. MPC	$81.10^{defghijklmn}$	86.00^{nopqr}	0.53^{BCD}
	3. Glycerol	$82.00^{cdefghi}$	90.19^{bcde}	0.51^{DE}
	4. Cocoa Butter	79.04^{qrstuv}	84.64^{rst}	0.76^{ijkl}
29	1. Control	82.67^{abcde}	87.59^{ijklm}	0.69^{pq}
	2. MPC	83.99^{a}	87.96^{hijkl}	0.58^{wx}
	3. Glycerol	$81.34^{cdefghijklm}$	89.66^{bcdef}	0.48^{F}
	4. Cocoa Butter	$81.02^{efghijklmno}$	84.96^{qrs}	0.76^{ijklm}
43	1. Control	$82.31^{abcdefg}$	87.39^{klmno}	0.71^{op}
	2. MPC	83.90^{ab}	90.49^{abcd}	0.51^{E}
	3. Glycerol	77.58^{vwx}	89.01^{defghi}	0.54^{zABC}
	4. Cocoa Butter	$82.42^{abcdefg}$	86.42^{mnopq}	0.77^{ghij}
60	1. Control	82.43^{abcdef}	88.65^{fghijk}	0.68^{q}
	2. MPC	83.00^{abc}	88.89^{efghij}	0.57^{wxy}
	3. Glycerol	76.50^{wxy}	87.61^{ijklm}	0.47^{F}
	4. Cocoa Butter	82.81^{abcd}	86.72^{lmnop}	0.66^{rs}
90	1. Control	83.91^{ab}	89.84^{bcdef}	0.70^{pq}
	2. MPC	84.00^{a}	89.12^{cdefgh}	0.47^{F}
	3. Glycerol	76.24^{xy}	89.56^{bcdefg}	0.44^{G}
	4. Cocoa Butter	$82.03^{cdefghi}$	87.34^{klmno}	0.68^{q}

[1]33.9% WPI, 46.7% sorbitol syrup, 19.4% vegetable shortening

$^{abc\ldots xyzABC}$ means within columns in Tables A.21 and A.22 with same letter were not significantly different, $\alpha = 0.05$

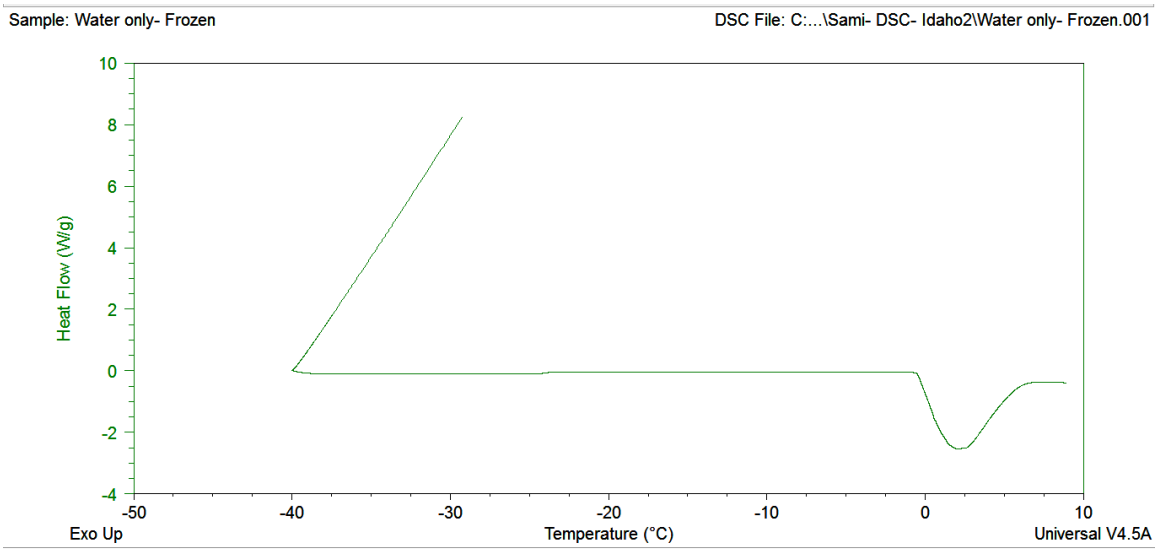

Figure A.1 Thermogram of water showing endothermic transitions for melting of bulk water ice crystals between 0 and 5°C (exo up).

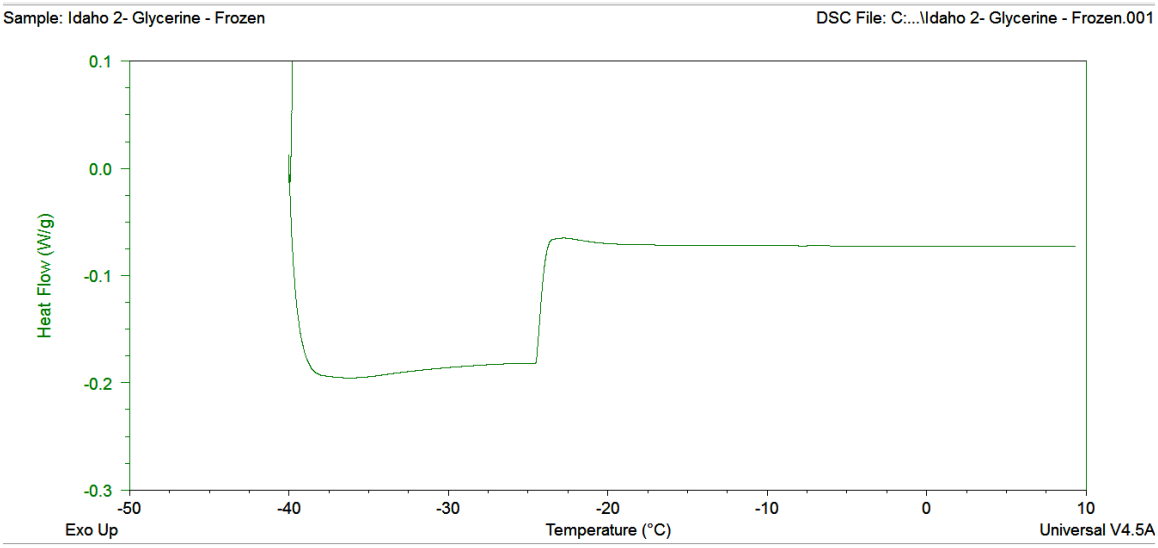

Figure A.2 Thermogram of glycerol showing step change in the thermogram at -24°C upon change in heating rate from 5°C/min to 2C/min and no further transitions at higher temperature in which melting of ice crystals associated with intermediate and bulk water occur (exo up).

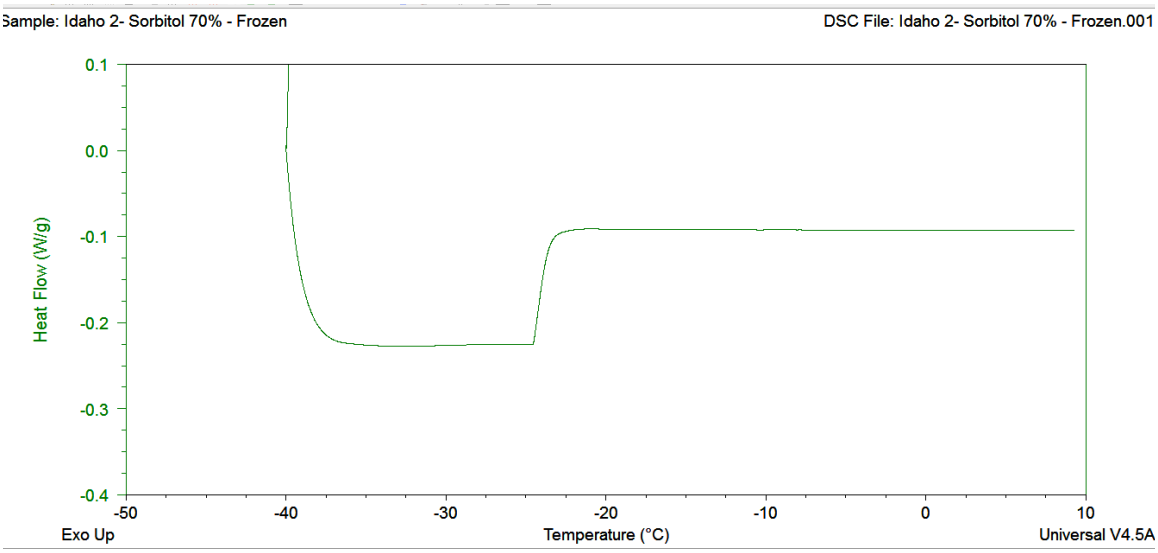

Figure A.3 Thermogram of sorbitol syrup showing step change in the thermogram at -24°C upon change in heating rate from 5°C/min to 2C/min and no further transitions at higher temperature in which melting of ice crystals associated with intermediate and bulk water occur (exo up).

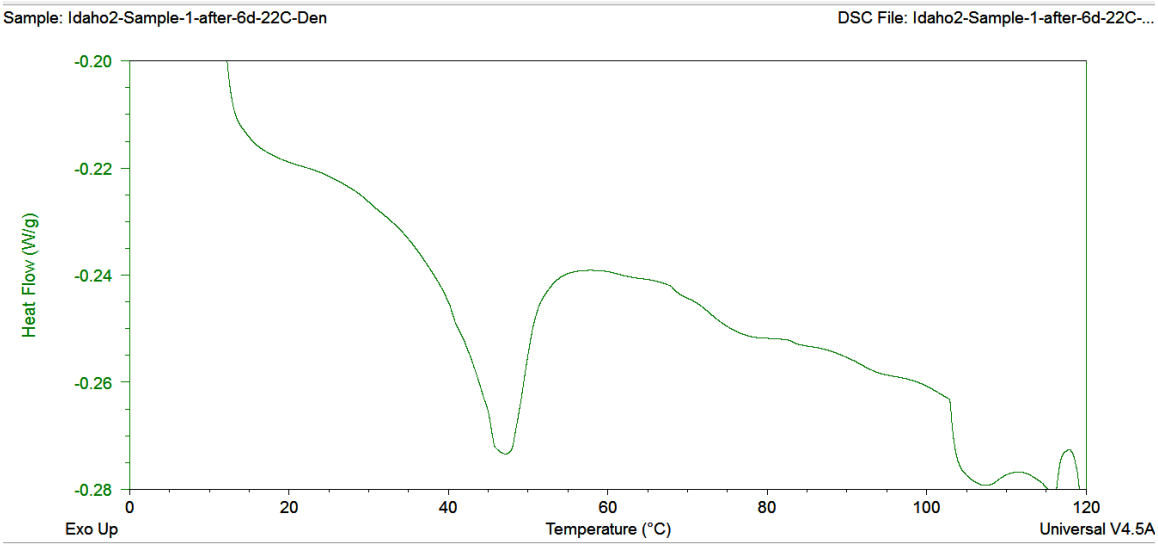

Figure A.4 Example of a differential scanning calorimeter thermogram of high-protein nutrition bars from 10°C to 120°C. The large endothermic transition between 40 and 50°C is melting of fat in shortening, the protein denaturation endothermic transitions α-lactalbumin and β-lactoglobulin occurred between 60 and 85°C (exo up).

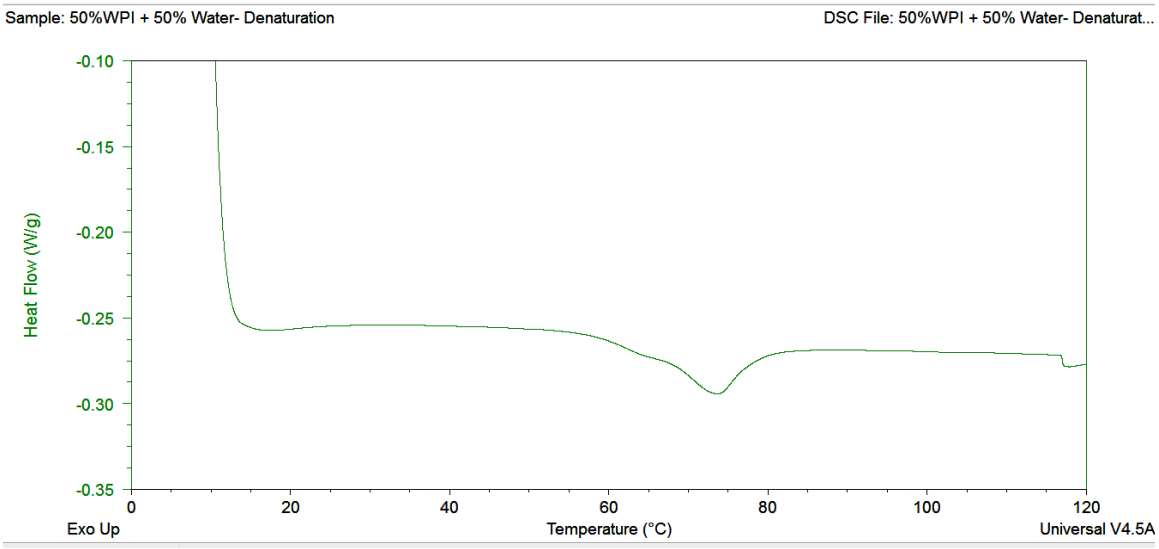

Figure A.5 Thermogram of 50% Whey protein isolate in water showing protein denaturation endothermic transitions α-lactalbumin and β-lactoglobulin occurring between 60 and 85°C (exo up).

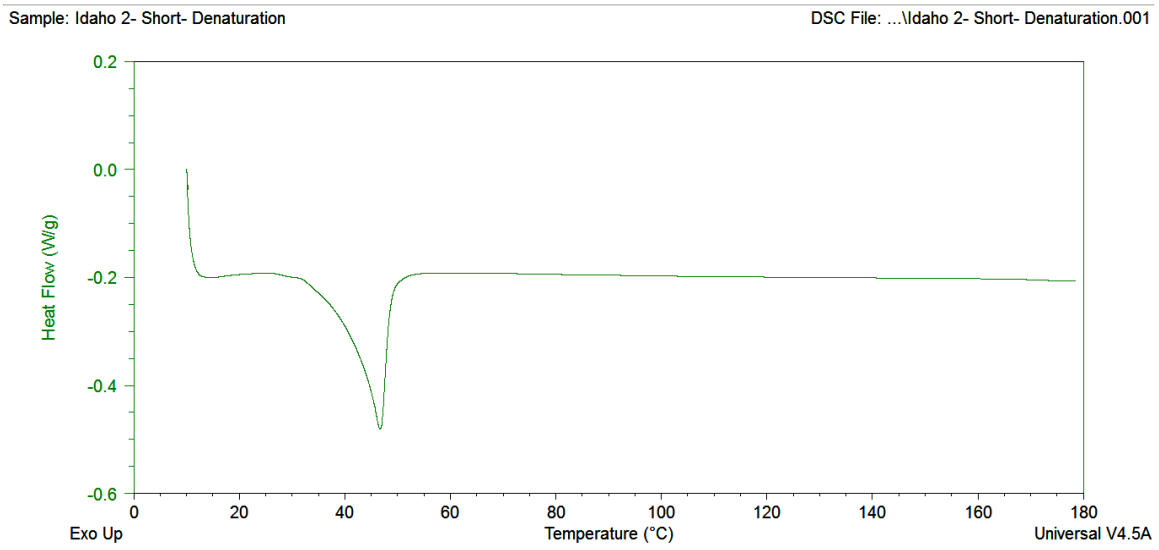

Figure A.6 Thermogram of vegetable shortening showing endothermic fat melting transitions between 40 and 50°C with no further transitions at higher temperatures where denaturation of α-lactalbumin and β-lactoglobulin occurs (exo up).

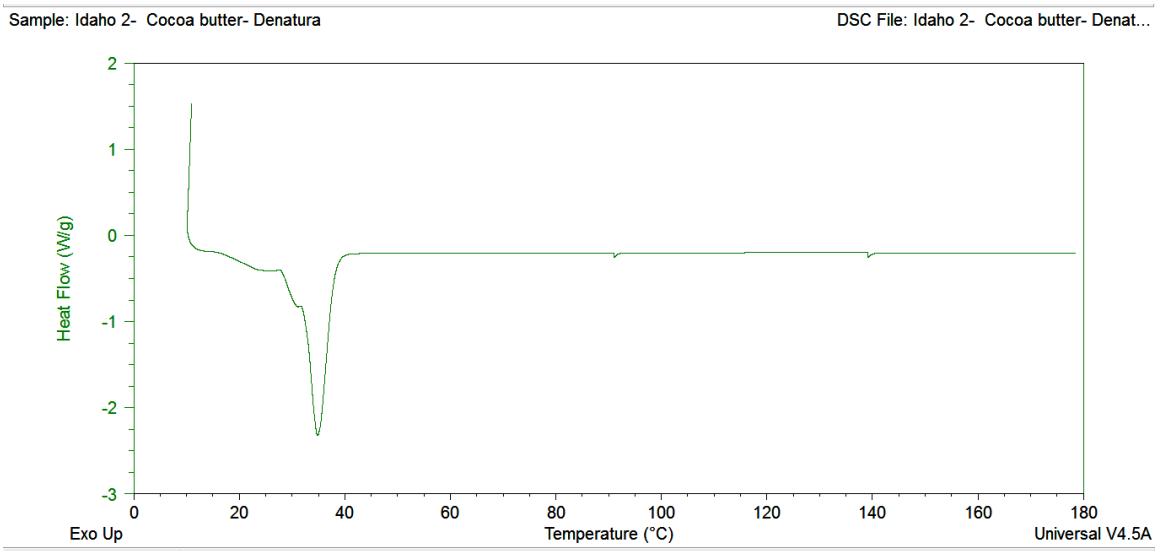

Figure A.7 Thermogram of cocoa butter showing endothermic fat melting transitions between 30 and 40°C with no further transitions at higher temperatures where denaturation of α-lactalbumin and β-lactoglobulin occurs (exo up).

APPENDIX B. PHASE 3 EXPERIMENTS

Table B.1 Color- L* of high-protein nutrition bars made using whey protein isolate (WPI) or a 50:50 WPI/milk protein concentrate (MPC) mixture, combined with shortening, sorbitol syrup, and glycerol (as described in Table 3.2) during 84 d of storage at 35°C.

Storage Time (d)	Color- L*			
	Sorbitol		Sorbitol+Glycerol	
	WPI (Bar A)	WPI/MPC (B)	WPI (C)	WPI/MPC (D)
1	89.4^{c}	91.8^{a}	89.8^{bc}	90.5^{b}
7	79.9^{k}	87.5^{d}	80.9^{j}	87.7^{d}
14	79.0^{lm}	86.3^{e}	79.7^{kl}	86.9^{de}
21	78.2^{mn}	85.5^{f}	77.5^{n}	86.6^{e}
42	77.9^{n}	84.0^{g}	76.5^{o}	85.4^{f}
63	76.5^{o}	83.0^{h}	76.0^{o}	84.0^{g}
84	74.5^{p}	81.8^{ij}	74.5^{p}	82.3^{hi}

$^{abc\ldots opq}$ means with same letter were not significantly different, $\alpha = 0.05$.

Table B.2 Color a* of high-protein nutrition bars made using whey protein isolate (WPI) or a 50:50 WPI/milk protein concentrate (MPC) mixture, combined with shortening, sorbitol syrup, and glycerol (as described in Table 3.2) during 84 d of storage at 35°C.

Storage Time (d)	Color- a*			
	Sorbitol		Sorbitol+Glycerol	
	WPI (Bar A)	WPI/MPC (B)	WPI (C)	WPI/MPC (D)
1	0.45^{hij}	0.01^{l}	0.36^{ijk}	0.07^{kl}
7	0.94^{g}	0.28^{jkl}	0.89^{g}	0.29^{jkl}
14	1.35^{f}	0.63^{ghi}	1.31^{f}	0.82^{g}
21	1.62^{ef}	0.71^{gh}	1.89^{e}	0.92^{g}
42	2.32^{d}	1.35^{f}	2.58^{cd}	1.74^{e}
63	3.62^{b}	2.27^{d}	3.40^{b}	2.81^{c}
84	4.20^{a}	3.32^{b}	3.98^{a}	3.56^{b}

$^{abc\ldots jkl}$ means with same letter were not significantly different , $\alpha = 0.05$.

Table B.3 Color b* of high-protein nutrition bars made using whey protein isolate (WPI) or a 50:50 WPI/milk protein concentrate (MPC) mixture, combined with shortening, sorbitol syrup, and glycerol (as described in Table 3.2) during 84 d of storage at 35°C.

Storage Time (d)	Color- b*			
	Sorbitol		Sorbitol+Glycerol	
	WPI (Bar A)	WPI/MPC (B)	WPI (C)	WPI/MPC (D)
1	11.1^{m}	10.6^{mn}	10.2^{n}	10.3^{mn}
7	16.3^{ij}	12.3^{l}	15.5^{j}	12.6^{l}
14	18.1^{h}	14.2^{k}	17.1^{i}	13.8^{k}
21	19.5^{g}	15.8^{j}	19.9^{fg}	15.8^{j}
42	21.9^{e}	19.4^{g}	22.7^{de}	20.6^{f}
63	25.7^{c}	23.2^{d}	24.9^{c}	25.2^{c}
84	29.1^{a}	28.1^{b}	28.1^{b}	28.4^{ab}

$^{abc\ldots lmn}$ means with same letter were not significantly different, $\alpha = 0.05$.

Table B.4 ANOVA of dependent variables for high-protein nutrition bar frozen onset T°C1, peak T°C1, onset T°C2, peak T°C2 values.

Effect	*P*				
	df	Onset T°C1	Peak T°C1	Onset T°C2	Peak T°C2
Carbohydrate (Carb)	1	0.0002	0.0003	<0.0001	0.7893
Protein (Prot)	1	0.0007	0.3867	0.0001	<0.0001
Carb x Prot	1	0.0071	0.0031	0.0001	0.4254
Time (T)	4	<0.0001	<0.0001	<0.0001	<0.0001
Carb x T	4	<0.0001	<0.0001	<0.0001	<0.0001
Prot x T	4	<0.0001	<0.0001	<0.0001	<0.0001
Carb x Prot x T	4	<0.0001	<0.0001	<0.0001	<0.0001

SAS Statistics Code used for Analysis of Phase 3 Data:

```
%let resp = loghardness; /*change onset1 to the response
name to be analyzed  */
proc glimmix data = color plots = studentpanel; /*need to
specify the library of the data. */
     class carbohyd protein time rep;
     model loghardness = carbohyd|protein|time;
     random int carbohyd*protein /sub = rep;
     lsmeans carbohyd*protein*time /slicediff=(carbohyd
protein time) adjust = tukey lines;
run;
```

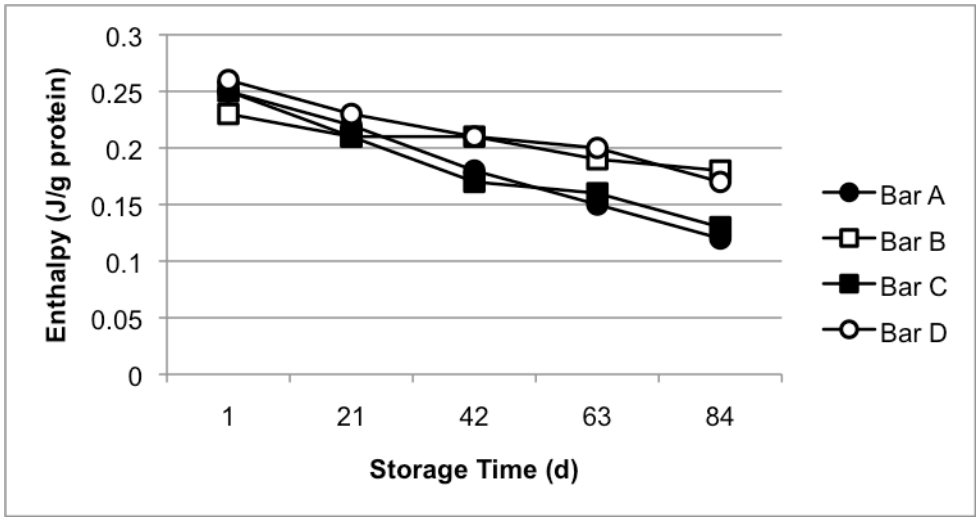

Figure B.1 Change in heat denaturation enthalpy of α-lactalbumin of high-protein nutrition bars made using whey protein isolate (WPI) or a 50:50 WPI/milk protein concentrate (MPC) mixture, combined with shortening, sorbitol syrup, and glycerol (as described in Table 3.2) during 84 d of storage at 35°C.

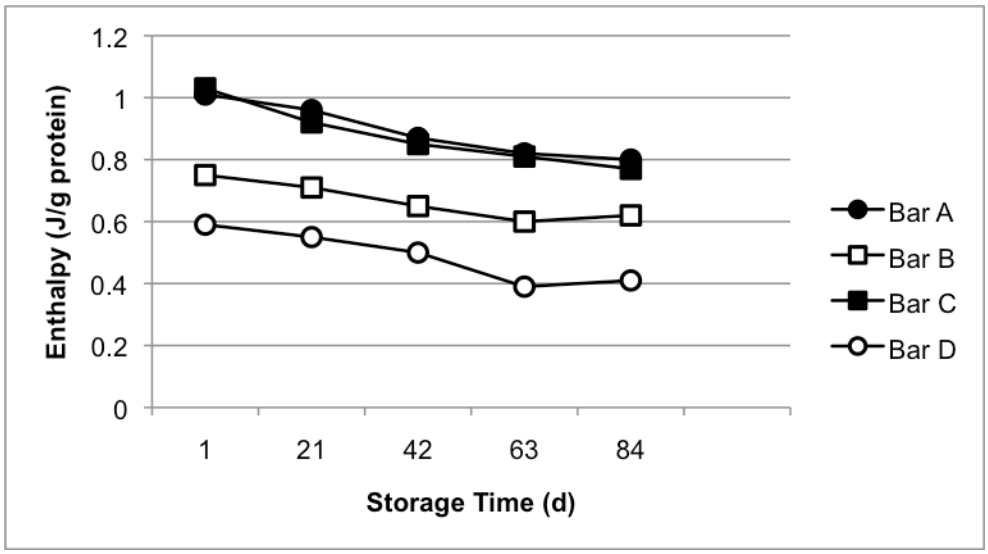

Figure B.2 Change in heat denaturation enthalpy of β-lactoglobulin of high-protein nutrition bars made using whey protein isolate (WPI) or a 50:50 WPI/milk protein concentrate (MPC) mixture, combined with shortening, sorbitol syrup, and glycerol (as described in Table 3.2) during 84 d of storage at 35°C.

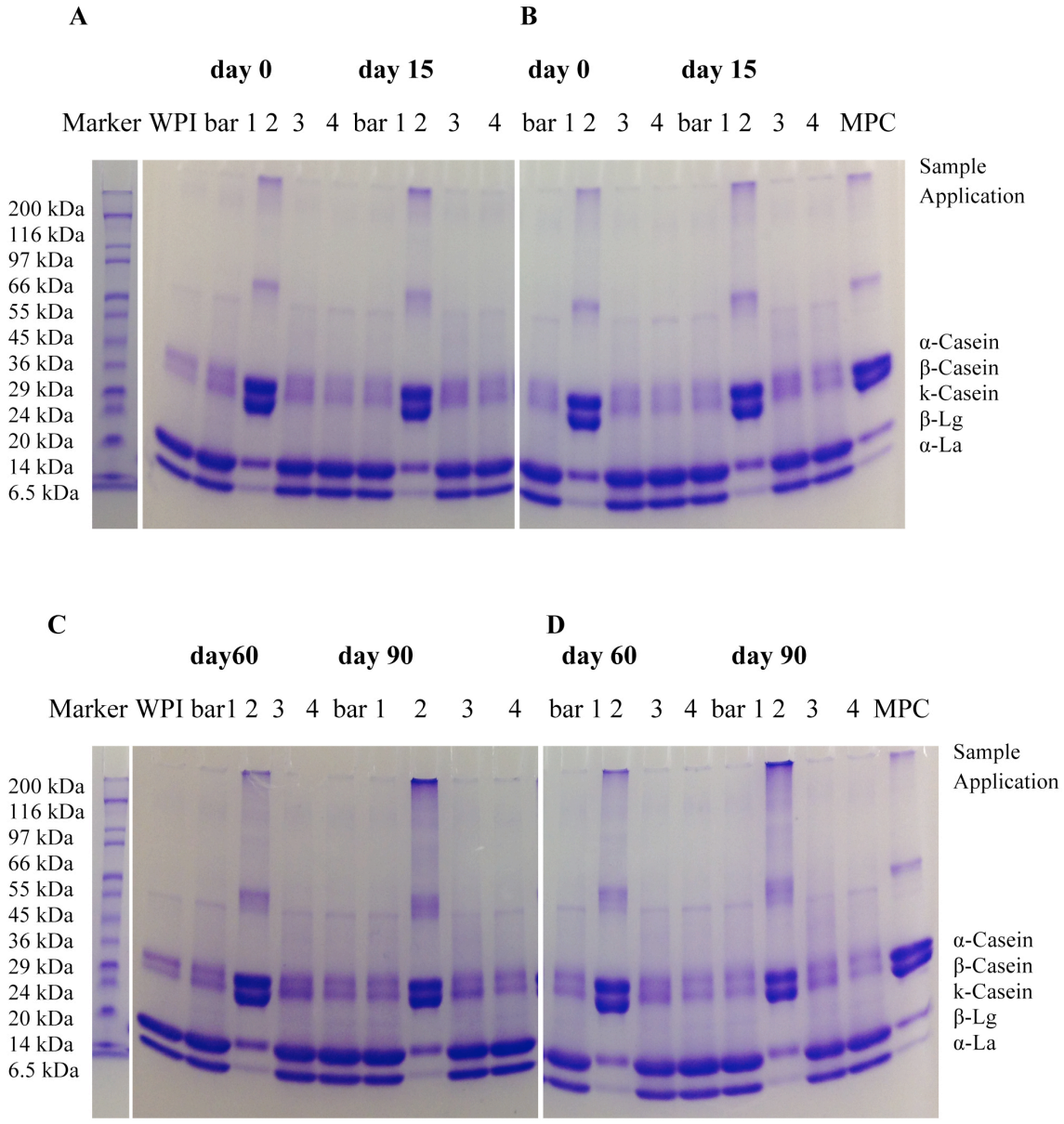

Figure B.3 SDS-PAGE using non-reducing and reducing conditions of Bars 1, 2, 3 and 4 from Chapter 5 under non-reducing condition (A and C) and after the sample was reduced using DTT (B and D) during 90 d storage at 35°C.

CURRICULUM VITA

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USU Invention Disclosure D15012 (2014): High-protein nutrition bars without hardening upon storage.

- Produced a new understanding in food chemistry related to the manufacture of high-protein nutrition bars.
- Letter of congratulations and thanks (No. 2081, September 16, 2015) from Embassy of the Republic of Iraq, Cultural Office, Representative of Iraqi Ministry of Higher Education & Scientific Research (MoHESR) in U.S.A.

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1. A study of the Effect Microwave Heating on Groundnuts phospholipids V:3: N:1: 1998.
2. A study of Ecological pollution in the Tigris (the Kut District) V:4: N:1: 1999.
3. Biological Influence of Industrial Wastes of the Two Textile and Rubber Factories on the Characteristics of Al-Diwaniya River Water V:5: N:1: 2000.
4. The Effect of Diwaniya Dairy Factory on Water pollution V:7: N:2: 2002.
5. The Effect of Sewage and Industrial Waste Waters on the Euphrates in Al-Samawa City. V:7: N:2: 2002.
6. Effect of the Different Methods of Heat Treatments on Groundnuts Saccharides V:7: N:3: 2002.
7. Effect of the Different Methods of Heat Treatments on Groundnuts Tocopherols V:9: N:1: 2004.
8. Effect of the Different Methods of Heat Treatments on Groundnuts Sterols V:9: N:3: 2004.
9. An Evaluation physical and Chemical Study of the Under Ground Water in the Middle Euphrates District V:10: N:1: 2005.

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